The Role of $VPAC_2$ Receptors and PKA in Neuropathic Pain

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Thesis presented for the degree of Doctor of Philosophy

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2001

DECLARATION

I hereby declare that the composition of this thesis and the work presented in it are entirely my own work, with the exception of the electrophysiological studies, which were carried out by Dr Gordon Blackburn-Munro and Dr Sue Fleetwood-Walker and ex vivo PKA activity assays which were carried out by Dr Rory Mitchell in the MRC Membrane and Adapter Protein Co-op, University of Edinburgh. Assistance and advice was kindly given by many individuals who are rightly acknowledged overleaf.

Some of the material from this thesis has been published:

Full Papers

Moss A, Blackburn-Munro G, Garry EM, Blakemore JA, Dickinson T, Rosie R, Mitchell R, Fleetwood-Walker SM A role of the ubiquitin-proteasome system in neuropathic pain. Journal of Neuroscience (In Press).

Abstracts

Moss A, Garry EM, O'Neill F, Dickinson T, Shen S, West K, Harmar AJ, Fleetwood-Walker SM (2001). Attenuation of neuropathic pain in $VPAC_2$ Receptor-null mice. Society for Neuroscience.

Moss A, Blackburn-Munro G, Garry EM, Blakemore JA, Mitchell R, Fleetwood-Walker SM (2001). The role of the ubiquitin-proteasome pathway in neuropathic pain. *BNA abstracts* **54.12**: P71.

Andrew Moss

ACKNOWLEDGEMENTS

This work was supported by the Wellcome Trust and a studentship awarded by the Medical Research Council. I would like to begin by thanking my supervisor Dr Sue Fleetwood-Walker for all her advice as well as her constant support. I would like to thank Dr Rory Mitchell from the MRC Membrane and Adapter Protein Co-op for his expert assistance with all things pharmacological. I would like to thank Professor Vince Molony and Dr Mike Shipston (thesis committee) and I would also like to thank Dr Roger Clegg and Kevin Connor from the Hannah Research Institute for their kind assistance and passing on their expertise.

I would also like to thank the members of my lab for teaching me all they know and making the three years immensely enjoyable. In particular, Emer Garry, James Blakemore, Vicky Wallace, Francis O'Neill, Gordon Blackburn-Munro, Tracey Dickinson and John Wilson. I would also like to thank everyone at the Department of Preclinical Veterinary Sciences for their expert assistance. My special thanks to Colin Warwick for his expert assistance in the Photographic Unit, Gordon Goodall and Jean Samuel in Histology and Stevie Mitchell in the Electron Microscopy Unit. I would also like to thank the MAFF Animal Unit, for all their help. In addition, my special thanks to Derek Penman for administrative assistance and Anne Stirling-White and Frances Fettes for secretarial support.

I am especially indebted to all my family and friends for their support and constant questions like "so when are you going to get a real job?" Finally, I would like to thank Kelly Conlon for helping me get through the thesis writing and job hunting and for her expert scientific guidance and patience.

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ABSTRACT

In animal models of neuropathic pain, changes in afferent and spinal cord neurones after peripheral nerve injury lead to hyperexcitability within the spinal dorsal horn, termed "central sensitisation". This causes a persistent pain state with enhanced responses to noxious stimuli (hyperalgesia) and pain-like responses to previously innocuous stimuli (allodynia).

The role of the $VPAC_2$ receptor in CCI was investigated. In $VPAC_2R^{(-/-)}$ mice, the enhanced reflex responses to noxious heat and innocuous mechanical stimulation seen in wild type (WT) mice were attenuated. No morphological differences were seen between peripheral nerves of WT and $VPAC_2R^{(-/-)}$ mice. Furthermore, intrathecal administration of a $VPAC_2R$ inhibitor attenuated the enhanced reflex withdrawal responses to noxious heat and innocuous mechanical stimuli in WT mice following CCI, with no effect in $VPAC_2R^{(-/-)}$ mice.

In normal rats, intrathecal administration of PKA inhibitors attenuated the enhanced reflex withdrawal responses due to CCI. In situ hybridisation for isoforms of PKA regulatory (R) and catalytic (C) subunits showed a spinal increase in C-subunit , but not R-subunit mRNA ipsilaterally at the peak of CCI sensitisation. Immunoblots confirmed an ipsilateral increase in C-subunits and showed a bilateral decrease in the RIβ subunit.

The role of the proteasome in neuropathic sensitisation was studied. Electrophysiological recordings made from dorsal horn neurones in anaesthetised rats showed that proteasome inhibitors applied by ionophoresis inhibited activity evoked by innocuous brush and cold in CCI rats, while nociceptive responses were inhibited in CCI and normal animals. Intrathecal administration of proteasome inhibitors attenuated the enhanced paw withdrawal behaviours ipsilateral to CCI. The mRNA and protein levels for UCH-L1, (a key enzyme in proteasomal function) were increased ipsilaterally. PKA enzymatic activity was increased in spinal cord ipsilateral to nerve injury and this increment was prevented by topical application of proteasome inhibitors.

This investigation demonstrates the involvement of the $VPAC_2$ receptor, the corresponding cAMP/PKA signal transduction cascade and the proteasome (a regulator of PKA activity) in the spinal sensitisation caused by CCI.

ABBREVIATIONS

AC	adenylate cyclase
ADP	adenosine diphosphate
AKAPs	A kinase anchoring proteins
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxadole proprionic acid
ANOVA	analysis of variance
Ap-UCH	*Aplysia* UCH
A/s	action potentials per second
ATP	adenosine triphosphate
°C	degrees celcius
C	PKA catalytic subunit
C	contralateral
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CCI	chronic constriction injury
cDNA	complementary DNA
CGRP	calcitonin gene related peptide
CNS	central nervous system
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
DAG	di-acyl glycerol
dATP	deoxyadenosine triphosphate
des-(1-4)-Arg^{16}-Ro 25-1553	(des 1-4)[Glu8, Lys12, Arg16, Nle17, Ala19] VIP (1-24) Asp, Leu, Lys, Gly, Gly, Thr, NH2, (lactam 21-25)
ddH2O	double distilled water
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
DTT	dithiothreitol
EAA	excitatory amino acid
ECL	enhanced chemi luminescence reagent
EDTA	ethylenediamine tetra acetic acid
ER	endoplasmic reticulum
G protein	guanyl regulatory protein
GABA	γ-aminobutyric acid
GAL	galanin
GAPDH	gluteraldehyde dehydrogenase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HCl	hydrochloric acid
HRP	horseradish peroxidase
5-HT	5 hydroxytryptamine
Hz	hertz
IP_3	inositol 1,4,5-triphosphate
I	ipsilateral
i.p.	intraperitoneal
i.t.	intrathecal

i.v.	intravenous
ISHH	in situ hybridisation histochemistry
kDa	kilodalton
l;ml	litre; mililitre
LTF	long term facilitation
LTP	long term potentiation
MAP-kinase	mitogen activated protein kinase
min	minutes
MΩ	mega ohms
Mg^{2+}	magnesium
mGluR	metabotropic glutamate receptor
M;mM	molar; milimolar
mg;kg	mili; kilogram
mN/mm^2	force in milinewtons per unit area (square millimetre)
mRNA	messenger RNA
msec	millisecond
N	normal
Na^+	sodium
nA	nano amps
NaCl	sodium chloride
NES	nuclear export sequence
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
nmol	nanomolar
NPY	neuropeptide Y
NRM	nucleus raphus magnus
$p \leq 0.05$	probability less than or equal to less that 0.05
PACAP	pituitary adenylate cyclase-activating polypeptide
PAG	periaqueductal grey
PBS	phosphate buffered saline
PF	paraformaldehyde
PGE_2	prostaglandin E2
PGP-95	protein gene product 9.5
PKA	protein kinase A
PKC	protein kinase C
PKI	protein kinase I
PLC	phospholipase C
PNL	partial nerve ligation
PSDC	post synaptic dorsal column
PVDF	polyvinylidene difluoride
R	PKA regulatory subunit
RNA	ribonucleic acid
RNase	ribonuclease
Ro 25-1553	[Ac-His1, Glu8, Lys12, Nle17, Ala19] VIP (1-24), Asp, Leu, Lys, Lys, Gly, Gly, Thr NH2 (lactam 21-25)
S	sham-operated
s	seconds
s.c.	subcutaneous

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ser	serine
SEM	standard error of the mean
SMP	sympathetically mediated pain
SMT	spinomesencephalic tract
SOM	somatostatin
SP	substance P
SPET	suspended paw elevation time
SPN	sympathetic postganglionic neurones
SRT	spinoreticular tract
SSC	standard saline citrate buffer
STT	spinothalamic tract
TdT	terminal deoxyribonucleotide transferase
thr	threonine
tRNA	transfer RNA
TTX	tetrodotoxin
UCH	ubiquitin carboxyl-terminal
UCH-L1	ubiquitin carboxyl-terminal hydrolase subtype 1
VIP	vasoactive intestinal polypeptide
vol	volume
$VPAC_2R^{(-/-)}$	Vasoactive intestinal polypeptide receptor type 2 knockout
wt	wild-type

CHAPTER 1: INTRODUCTION

1.1 General Introduction

One of the functions of the nervous system is to provide information about the occurrence or threat of injury. The sensation of pain, by its inherent aversive nature, contributes to this function. Chronic pain due to nerve injury, termed neuropathic pain, leads to the development of abnormal pain sensations which can in some cases last from months to decades (Scadding, 1984). The pathological conditions often persist long after healing of the damaged peripheral tissue or nerve and are thought to be indicative of a dysfunctional nervous system. This thesis is focused on neuropathic pain since its physiological basis is poorly understood and little is available as a satisfactory treatment. Clinically it represents a substantial problem, with approximately 1.5% of the U.S. population estimated to be afflicted and there are currently no effective and acceptable analgesic regimens to treat these individuals. Before discussing the specific mechanisms of neuropathic pain, and focusing on potential targets, it is important to consider the anatomy, physiology and pharmacology of nociceptive processing in the peripheral and central nervous system. This should facilitate recognition and understanding of the changes, which occur both peripherally and centrally following nerve injury. The thesis will then focus on the central changes that occur in the spinal dorsal horn following peripheral nerve injury.

1.2 Fibre Composition of Cutaneous Nerves

There are various groups of primary afferent fibres, classified according to their axon diameter, conduction velocity and whether they are myelinated or not. The largest group of nerve fibres in cutaneous nerves is the myelinated A-fibre class. The rat sciatic nerve contains approximately twice as many of these myelinated sensory axons compared to unmyelinated fibres (Schmalbruch, 1986). Non-noxious inputs from the cutaneous surface are mediated principally by large myelinated (Aβ) fibres which have conduction velocities of greater than 30m/s. Noxious inputs are mediated by fine afferent fibres, both myelinated Aδ and unmyelinated C fibres, with

average conduction velocities of 5-30m/s and 0.5-2m/s respectively (Willis and Coggeshall, 1991).

1.3 Classification of Cutaneous Sensory Receptors of Afferent Nerves

The terminal regions of the primary afferent fibres, found in the peripheral tissue, constitute the receptive or dendritic part of the neurone, which have contact with well defined areas of skin from which the afferent fibres can be excited (receptive field). Cutaneous receptors transmit information about the external environment for example (heat, pressure, chemicals). There are clearly distinguishable classes of specialised nociceptors and mechanoreceptors (Lynn, 1994).

1.3.1 Non-Nociceptive Mechanoreceptors

Cutaneous mechanoreceptors are the most sensitive receptors, responding most readily to mechanical pressure stimuli of various intensities. They can be further subdivided according to their other characteristics.

1.3.2 Hair Follicle Receptors

These receptors are units that respond to hair movement. They are the predominant class of units with myelinated Aδ axons within rat peripheral nerves (Lynn and Carpenter, 1982). They have been classified according to the hairs they innervate (Brown and Iggo, 1967). D-hair units are rapidly adapting receptors, typically activated by movement of down hairs but also responding to movement of guard hairs. They are supplied by nerve fibres ~4μm in diameter with conduction velocities from 12-30 m/s. Each fibre innervates many down hairs and have large receptive fields (~2cm^2). G-hair units are rapidly adapting receptors associated with guard hair follicles only, each nerve fibre supplying ten or more follicles. These receptors are termed "basket endings" since the terminals surround the hair in a network that resembles a wicker basket. The afferent fibres are 5-15μm in diameter, have conduction velocities from 20-90m/s and are activated by relatively fast movement of the guard hairs (Lynn and Carpenter, 1982). T-units are the least

numerous and tend to have high conduction velocities (mean about 65 m/s) and are activated by movement of the large tylotrich hairs (Lynn and Carpenter, 1982).

1.3.3 Field Receptors, Rapidly Adapting (RA) Cutaneous Receptors, Pacinian Corpuscles and Slowly Adapting (SA) (SA type I and II) Mechanoreceptors.

In glabrous skin there are two types of rapidly adapting (Meissner's and Pacinian corpuscles) as well as two types of slowly adapting (Merkel's and Ruffini corpuscle) touch receptors. The rapidly adapting cutaneous receptors respond to light pressure, stroking or vibration of the receptive field area. They are predominantly associated with large Aβ fibres and generally transmit only non-nociceptive information. Slowly adapting (SA type I or II) mechanoreceptors are also associated primarily with large Aβ fibres. SAI mechanoreceptors are low threshold receptors associated with Merkel cell complexes in the epidermis. SAII mechanoreceptors are identified with Ruffini endings located in the dermis and so respond to small displacements of the skin usually as a result of the skin stretching (Willis and Coggeshall, 1991).

1.3.4 Low Threshold C-Mechanoreceptors

These unmyelinated C fibres are highly responsive to gentle mechanical stimulation such as light brushing (Bessou and Perl, 1969). They constitute between 15-30% of C fibres in peripheral nerves. They have a small receptive field primarily located in hairy skin and have been shown to respond to sudden cooling of the receptive field area (Bessou and Perl, 1969; Lynn and Carpenter, 1982; Leem et al., 1993).

1.3.5 Non-Nociceptive Thermoreceptors

Thermoreceptors transmit innocuous changes in temperature, and are generally unresponsive to mechanical stimuli. They can generally be classified into two types:

1.3.6 Cold Thermoreceptors

Innocuous cool sensations and cold pain are mediated by different populations of primary afferent fibre. Cool sensations are signalled by activity in cold specific Aδ-fibres (and to a lesser degree cold-specific C fibres) (Iggo, 1959; 1969), with specific cutaneous receptors in both the hairy and glabrous skin (Iggo, 1969). Cold thermoreceptors are characterised by their high sensitivity to small falls in skin temperature (as little as 0.1°C), and the most commonly studied in the rat are facial and scrotal thermoreceptors. The majority of these receptors have a relatively restricted range of innocuous cold temperatures (approximately 20-30°C) over which they give dynamic responses to small reductions in skin temperature (Heinz et al., 1990; Iggo, 1969).

1.3.7 Warm Thermoreceptors

Warm thermoreceptors respond to slight warming of the skin and are generally thought to be unmyelinated (Iggo, 1959). They are active at normal skin temperature (approximately 30°C) and are silenced by noxious levels of heat (+48°C).

1.4 Nociceptors

The earliest reports on single unit activity in peripheral nerves predicted the presence of nerve fibres responsive to tissue threatening or tissue damaging stimulation (Zottermann, 1933). However, it is only since detailed studies in the late 1960's that evidence for the existence of specific cutaneous nociceptors in numerous species has accumulated (Burgess and Perl, 1973, Price and Dubner, 1977). Nociceptors can be classified according to their responses to different forms of intense stimulation and the conduction velocity of their peripheral axons. There are two main groups of cutaneous nociceptors, the Aδ mechanical nociceptor and the C-polymodal nociceptor. Microneurography and intraneural microstimulation of identified cutaneous primary afferent fibres in humans demonstrated that electrical stimulation of nociceptive Aδ fibres evoked a sensation of sharp pain, whereas stimulation of

nociceptive C fibres produced a sensation of dull or burning pain (Torebjork and Ochoa, 1980; Ochoa and Torebjork, 1989).

1.4.1 Aδ Fibre Nociceptors

Aδ nociceptive fibres exist in both the glabrous and hairy skin and are mainly excited by high threshold mechanical stimulation. The majority of Aδ nociceptive fibres have been termed high threshold mechanoreceptors (HTM). Aδ nociceptive primary afferents originating from high threshold mechanoreceptors terminate predominantly in laminae I, IV and V of the dorsal horn (Burgess and Perl, 1973). A distinctive feature of HTMs is the change in responsiveness produced by repetitive heat stimulation (Burgess and Perl, 1973). Although rarely responsive to initial heat stimuli in the 45°C-55°C range, HTMs often respond to heat after repeated stimulation, a phenomenon termed "sensitisation" (Bessou and Perl, 1969). Leem et al. (1993) classed 70% of Aδ nociceptors as mechanical nociceptors, 20% as mechanoheat nociceptors and 10% as mechanocold nociceptors. Recent work by Simone and Kajander, (1996; 1997) have shown that all mechanosensitive Aδ nociceptors responded to noxious cold stimuli. It should be noted that activation thresholds for the majority of Aδ nociceptors was < 0°C and the physiological relevance of these interesting findings are unclear.

1.4.2 C Fibre Nociceptors

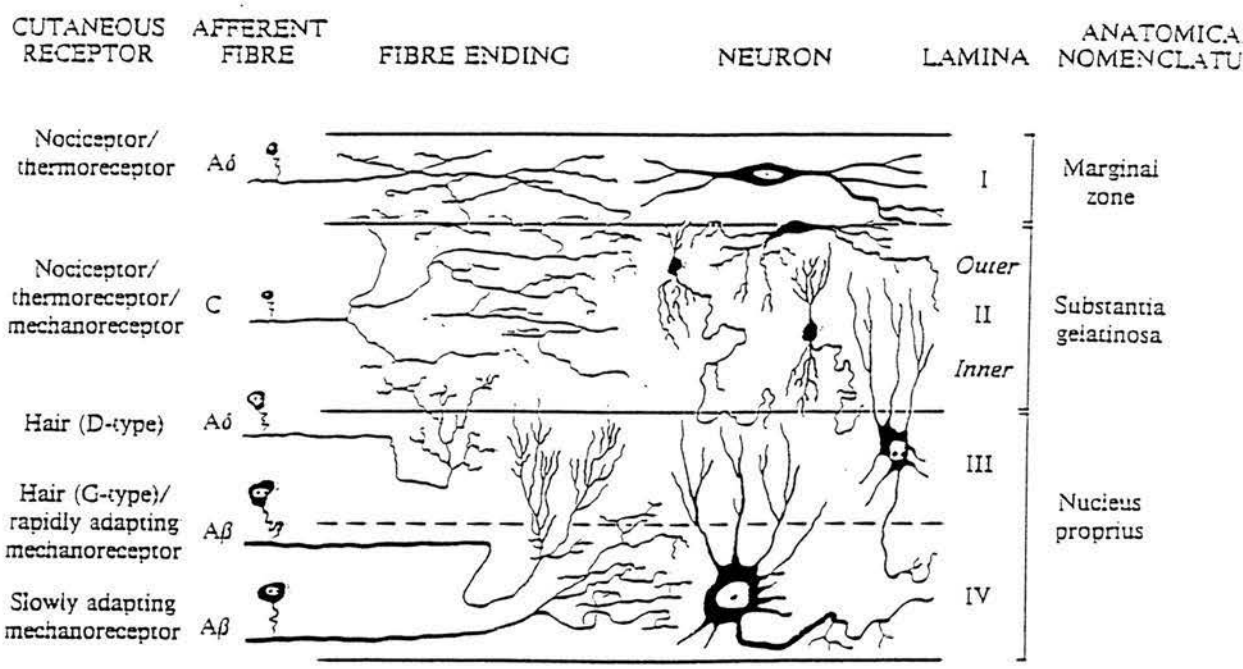
Fibres which respond to noxious thermal, mechanical, cold and chemical stimuli have been called polymodal nociceptors. Following classification by Burgess and Perl (1973) C fibre nociceptors are referred to as C-polymodal nociceptors (CPNs). CPNs are the most abundant form of primary afferent C fibre found within peripheral nerves of the rat (Lynn and Carpenter, 1982; Schmalbruck, 1986). They exist in the glabrous and hairy skin and typically have small receptive fields. CPNs generally respond to heat at initial temperatures of 42°C-55°C (ranging from borderline to overtly noxious and damaging). CPNs are also excited by irritant or caustic chemical agents applied to the skin and typically respond to high threshold mechanical

stimulation (Perl, 1984). CPNs are excited by a wide range of cold stimuli, the only difference in cold evoked responses of Aδ and CPNs is in their response thresholds. CPNs response threshold is lower and generally >0°C. CPNs have a characteristically low background level of activity unless the skin has been previously noxiously stimulated. The vast majority of cutaneous CPNs have been shown to terminate in laminae I-II of the spinal dorsal horn (Cervero and Iggo, 1980; McMahon et al., 1984).

Figure 1.1 Schematic Diagram of the Cutaneous Afferent Input to, and Neuronal Organisation of the Spinal Dorsal Horn of the Cat

A hypothetical cross section of the spinal dorsal horn, illustrating the afferent fibres and neuronal elements presented in the first four laminae. The laminar divisions of Rexed (1952) are indicated on the right. Afferent fibre types are listed to the left of the diagram, shown projecting onto neuronal types typical of laminae I-IV. The neurones illustrated here are (from top to bottom): a marginal cell, an SG limiting cell, two SG central cells and two neurones of the nucleus proprius, the more superficial of which has dendrites penetrating lamina II.

(Taken from Cervero and Iggo, 1980)


CUTANEOUS RECEPTOR
AFFERENT FIBRE
FIBRE ENDING
NEURON
LAMINA
ANATOMICA NOMENCLATU
Nociceptor/ thermoreceptor
Aδ
Nociceptor/ thermoreceptor/ mechanoreceptor
C
Hair (D-type)
Aδ
Hair (G-type)/ rapidly adapting mechanoreceptor
Aβ
Slowly adapting mechanoreceptor
Aβ
I
Outer
II
Inner
III
IV
Marginal zone
Substantia gelatinosa
Nucleus proprius

1.5 Laminar Organisation of the Dorsal Horn of the Spinal Cord

The grey matter of the spinal cord, which comprises nerve cell bodies, fibres (axons and dendrites) and associated non-neuronal cells can be grossly divided into two main sub-divisions, the anterior or ventral horn and the posterior or dorsal horn (Figure 1.2). These distinct anatomical characteristics were first documented by Rexed, (1952) who examined the feline spinal cord and classified the laminae according to their cryoarchitectonic characteristics. Further anatomical studies have demonstrated a similar architectonic scheme in the rat (Molander et al., 1984). According to Rexed's nomenclature, the dorsal horn of the spinal cord is sub-divided into 6 distinct laminae.

1.5.1 Lamina I (LI, The Marginal Zone)

LI is the most superficial layer of the dorsal horn. It covers the dorsal surface of the dorsal horn, bends around its apex and extends about halfway down the lateral side (Molander et al., 1984). It is the thinnest of the laminae and contains small medium and large neurones. Cells are generally elongated and spindle shaped. The largest are very elongated (30-50μm x 10-15μm, Rexed, 1952), and are termed the marginal cells of Waldeyer (1888). The marginal cells in LI send dendrites out over the surface of the dorsal horn, their disc like dendritic domains remaining within lamina I (Ralston, 1968). As well as these cells LI contains other cells, which merge with the outer layer of LII (Lima and Coimbra, 1983). In LI the orientation of neuronal processing is parallel to the dorsal border of the horn in the transverse plane (Ralston, 1968). Both unmyelinated C fibres and myelinated Aδ- fibres terminate in LI with the thinly myelinated Aδ fibres predominating (Willis and Coggeshall, 1991). There is no evidence of the large myelinated Aβ fibres terminating in LI (Brown, 1981). A number of reports suggest LI contains primarily noci-specific neurones which receive their projections from cutaneous, high threshold Aδ mechanoreceptors and C fibre thermal nociceptors (Cervero et al., 1976; 1979; Christensen and Perl, 1970; Light and Perl, 1979; Rethelyi et al., 1983). However, more recent studies have revealed a large proportion of rat LI cells to be multireceptive (McMahon and Wall, 1983; Menetrey and Besson. 1981). Overall then, the vast majority of lamina I

dorsal horn neurones have the ability to respond to nociceptive stimuli, highlighting the importance of LI in the transmission of noxious sensory information.

1.5.2 Lamina II (LII, The Substantia Gelatinosa)

Lamina II is well defined due to its gelatinous appearance. LII passes across the dorsal horn around the apex and down laterally. Covered dorsally and laterally by LI it has been described as the substantia gelatinosa (Rolando, 1824). LII contains tightly packed small cells ranging from (5 - 10μm in diameter). This lamina is believed to receive input primarily from unmyelinated primary afferents (Brown, 1981; Light and Perl, 1979; Cervero and Iggo, 1980), A combination of degeneration and Golgi staining experiments have demonstrated that many C fibre afferents terminate in this lamina (Lamotte, 1977; Rethelyi, 1977; Ralston and Ralston, 1979), with PHA-L injection of single fibres, confirming this (Sugiura et al., 1989). In contrast to LI, in LII the dendritic organisation is essentially in the long axis of the spinal cord (Sterling and Kuypers, 1967; Scheibel and Scheibel, 1968; Rethelyi and Szentagothai, 1969). The dendrites in LII extend longitudinally through LII and LIII in the saggital plane, but are severely restricted in the transverse plane. In contrast, the axons of LII cells are confined for the most part within LII (Matsushita, 1969). LII has recently been further sub-divided into LII inner (LIIi) and LII outer (LIIo) with LIIo the most superficial containing a high concentration of very fine primary afferent fibres. LIIi is the more deeply positioned area of LII with less densely packed neurones (Light and Perl, 1979).

1.5.3 Lamina III – IV (LIII-LIV, The Nucleus Proprius)

LIII is broader than both LI and LII and runs parallel to them. The cells are less tightly packed than LII (Light and Perl, 1979) and generally larger, with a few being much larger although these may belong in LIV (Rexed 1952). LII-IV contains relatively large cells ranging from (7-8μm x 10-12μm). Myelinated axons terminate here and pass through the lamina both rostrally and caudally. LIII receives direct inputs from both Aδ and Aβ fibres (Brown et al., 1977; Light and Perl, 1979). Lamina IV is a relatively thick layer, which also extends from the white matter of the

dorsal columns medially to where the ventral bend of LI-III runs down the lateral side of the dorsal horn. It is the first layer not to show the lateral bend. There is a less dense cellular distribution than LIII due to the large numbers of nerve fibres passing through it, and there is a huge heterogeneity of neurone size, ranging from very small to very large cells (Molander et al., 1984). It is predominantly the intermediate to thick diameter A-fibres which innervate these deeper dorsal horn laminae, including non-nociceptive Aδ fibres, originating from D-type hair follicles, and the large diameter Aβ axons of sensory, cutaneous mechanoreceptors (Brown and Iggo, 1967; Light and Perl, 1979).

1.5.4 Lamina V-VI (LV-LVI)

LV extends straight across the grey matter of the dorsal horn from the white matter of the dorsal columns medially to the white matter of the lateral columns. Some nociceptive (Aδ) afferents terminate in LV and both nocispecific and mulitreceptive neurones are found in this region (Kumazawa and Perl, 1977; Menetrey et al., 1977; Rethelyi et al., 1982). LV is divided into medial and lateral zones. The lateral zone contains more larger and fewer medium and small cells than the medial zone. In the lateral part of LV, the fibre bundles are large and numerous, giving the section a reticulated appearance (Light and Perl, 1979). LV contains a wide range of cell sizes ranging from 8μm x 10μm - 30μm x 40μm. The final lamina of the dorsal horn, LVI, has a slightly curved ventral edge. Similar to lamina V, there are large cells, but the lamina is dominated by the large number of small neurones in the medial zone (Willis and Coggeshall, 1991; Rexed, 1952; Molander et al., 1984). Both of these layers receive direct inputs from large Aβ fibres (Molander et al., 1984).

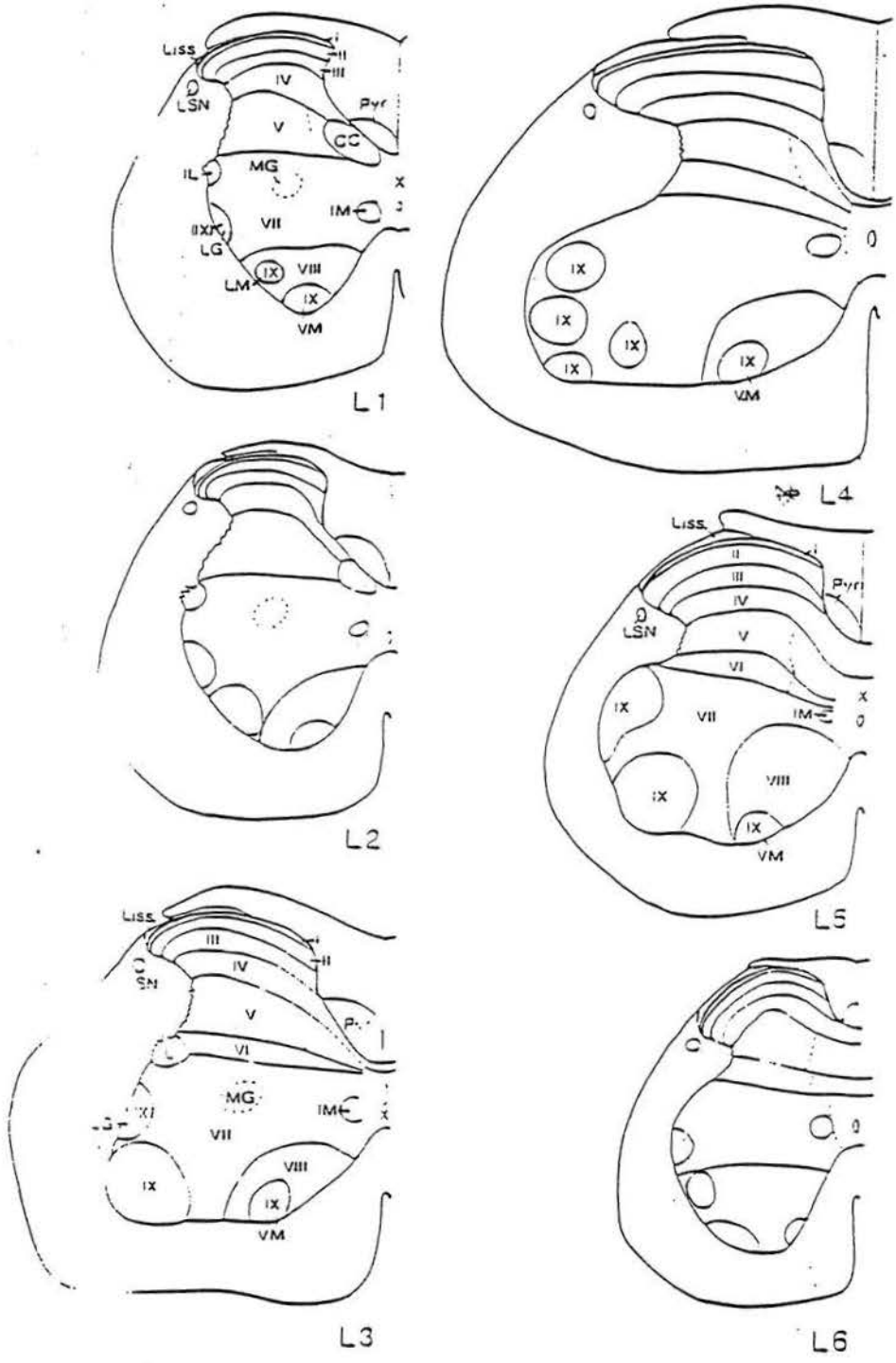
There are ten laminae in total named by Rexed (1952) and Molander et al. (1984) although the remaining laminae VII-X, represent the ventral horn and are beyond the scope of this study. Despite the distinct lamination pattern shown in figure 1.2 it is important to note that laminae are recognised primarily as zones of concentrations of particular cell types and may have intermingled edges. Also the borders between the laminae may differ between different segmental sections (Molander et al., 1984).

Figure 1.2 Schematic Diagram of the Cytoarchitectonic Organisation of Segments LI-VI of the Rat Spinal Cord

Representation of the laminar divisions of Rexed (1952) as demonstrated in the lumbar (L) segments of the rat spinal cord.

Abbreviations:
I – X – spinal cord laminae; CC – column of Clarke; IL – intermedio-lateral nucleus; IM – intermedio-medial nucleus; LSN – lateral spinal nucleus; Liss – Lissauer's tract; LG – lateral group of large cells in the dorso-lateral part of the ventral horn; LM – latero-medial nucleus; MG – medial group of large neurones in the intermediate zone; Pyr – pyramidal tract; VM – ventro-medial nucleus.
Note that LG, LM and VM are parts of lamina IX.

(Molander et al., 1984)


Liss
II
III
IV
LSN
Pyr
V
CC
MG
IL
IM
VII
LG
VIII
LM
IX
VM
L1
L2
L3
L4
L5
L6

1.6 Classification of Sensory Neurones

The spinal dorsal horn represents an important site for the initial processing of sensory information from the periphery to the CNS, especially with regards the transmission and modulation of pain. The dorsal horn is characterised by a wide range of cells of varying sizes and characteristics situated in specific layers, which can be classified according to their responsiveness to evoked sensory stimuli (Iggo, 1974).

1.6.1 Non-Nociceptive Neurones (Class I)

These cells are innervated by sensitive mechanoreceptors and respond only to innocuous mechanical stimulation of the cutaneous receptive field (Dubner and Bennett, 1983).

1.6.2 Multireceptive Neurones (Class II)

Also known as wide dynamic range neurones (WDR) (Mendell, 1966), these have convergent inputs arising from both peripheral nociceptors and sensitive mechanoreceptors, and therefore respond to both innocuous and noxious mechanical stimulation of the cutaneous receptive field as well as noxious thermal stimulation (Price et al., 1976; 1978). Wide dynamic range neurones tend to be situated in the deeper laminae of the dorsal horn (LIV-V) (Besson and Chaouch, 1987). However, they have also been reported in the superficial laminae (Iggo, 1974; Menetrey and Besson, 1981; McMahon and Wall, 1983; Woolf and Fitzgerald, 1983).

1.6.3 Nocispecific Neurones (Class III)

Nocispecific neurones are classified as a population of neurones that receive their inputs solely from nociceptive afferents (Aδ and C - Fibres) and therefore only respond to high threshold, noxious stimuli (Cervero et al., 1976). A relatively high proportion of nocispecific neurones have been reported in lamina I, including cells

which project to thalamic and brain stem regions (Cervero et al., 1976; 1979; Christensen and Perl, 1970; Light and Perl, 1979).

The location and type of neurones in the laminae of the dorsal horn will clearly influence the transmission of nociceptive information. In addition, the projection pattern of the different primary afferent fibre types will influence the type of response made by these dorsal horn neurones in different laminae, in particular the multireceptive neurones. Multireceptive dorsal horn neurones within the more superficial laminae of the dorsal horn are believed to be predominantly involved in the transmission of nociceptive information, since cutaneous Aδ and C fibres terminate primarily in lamina I and II respectively. In contrast, Aβ fibres project into the deeper dorsal horn (laminae III-IV). This preferential distribution rather than a strict lamination supports the laminar model of the dorsal horn being "zones of specialisation rather than absolute separate laminae of distinct specialisation" (Menetrey and Besson, 1981).

1.7 Ascending Somatosensory Pathways

As well as receiving descending inhibitory inputs from higher centres of the CNS, projection neurones ascend through distinct tracts in the spinal white matter and relay sensory information to regions of the brain stem and thalamus. The main ascending tracts implicated in the transmission of nociceptive information in the rat spinal cord are:

1.7.1 Spinothalamic Tract (STT)

The STT has been the subject of interest since its first description in the mammal by Mott (1895). Neurones in the spinal dorsal horn project to thalamic nuclei via the ventrolateral quadrant of the spinal cord, usually contralateral to the side of sensory input (Willis et al., 1979). Human studies have shown that lesions in this quadrant may lead to analgesia (Spiller and Martin, 1912), whereas stimulation may result in sensations that are painful (Mayer et al., 1975). Antidromically identified cells of the rat and primate STT were found to be widely distributed throughout the lumbar

dorsal horn of the spinal cord arising primarily from cell bodies located in lamina I, IV, V and VI (Dilly et al., 1968; Giesler et al., 1976). A wide range of somatosensory information is transmitted in the STT of the rat and primate, including responses to light touch, hair displacement, pressure, joint movement, heat and pinch (Willis et al., 1983). Willis et al., (1983) characterised the neurones of the STT in the monkey, on the basis of their responses to peripheral stimuli to reveal a high proportion of STT neurones respond to noxious stimulation (55% were multireceptive, while 32% were nocispecific). Interestingly, STT neurones located within the ventral horn that appeared to respond to stimuli delivered to subcutaneous tissue, tended to be activated from midline thalamic structures. In contrast, STT neurones within the nucleus proprius and marginal zone, which responded to noxious and innocuous cutaneous stimuli, were activated from lateral thalamic structures (Giesler et al., 1976).

1.7.2 Spinoreticular Tract (SRT)

The SRT projects from the dorsal horn of the spinal cord to the ventrolateral quadrant to the brainstem reticular formation which in turn relays information to the thalamus (Kevetter and Willis, 1983). Horseradish peroxidase (HRP) labelling has shown that Laminae VII and VIII cells in the rat ventral horn are the main origin of SRT cells, although a minority of cells appear to originate in the more superficial layers (Chaouch et al., 1983). Activation of SRT neurones by reticular formation stimulation revealed it was possible to activate neurones in laminae III-IV, and a few in laminae I, V and VI. In this study they observed almost half of the cells to be multireceptive and a further fifth to be nocispecific (Menetrey et al., 1980).

1.7.3 Spinomesencephalic Tract (SMT)

SMT neurones project from Lamina I predominantly and Lamina V to the mesencephalic reticular formation and lateral periaquaductal grey matter (PAG) (Mehler et al., 1960; Menetrey et al., 1982; Harmann et al., 1988). Whilst there are SMT cells that respond only to innocuous mechanical stimuli (Menetrey et al., 1980), electrophysiological studies have shown that a high proportion of SMT neurones,

originating in the marginal zone, are nociceptive (Menetrey et al., 1980), implicating the involvement of the SMT in the control of nociception and pain.

1.7.4 Post-synaptic Dorsal Column (PSDC)

The PSDC originates primarily from neurones on the Lamina II-III border of the rat spinal dorsal horn and projects through the dorsal funiculus to the nucleus gracilis and nucleus cuneatus (Giesler et al., 1984). Electrophysiological experiments have identified both multireceptive and nocispecific neurones in the PSDC (Angaut-Petit, 1975; Brown et al., 1983).

The STT, SRT, SMT and PSDC systems are the best understood, but not the only possible pathways for nociception. The spinocervical projection, for example may be involved in some aspects of the pain experience (Brown et al., 1979; 1977; 1980).

1.8 Functional Regulation of Dorsal Horn Neurones

1.8.1 Descending Control of Nociceptive Transmission

The existence of a specific pain modulatory system was first described clearly in 1965 by Melzack and Wall in the "Gate Control Theory" of pain. Supraspinal influences on the "gate" were proposed but there was limited evidence for descending control of nociception. In 1965, Melzack and Wall demonstrated that structures in the brainstem tonically inhibit nociresponsive neurones in the spinal cord.

Dorsal horn transmission is under the control of inhibitory mechanisms driven by mid-brain descending projections and segmental projections which synapse directly or indirectly onto primary afferent terminals, dorsal horn projection neurones and interneurones which can pre-synaptically reduce transmitter release from primary afferent terminals or hyperpolarise dorsal horn neurones. Both of these actions reduce the likelihood of dorsal horn neurone firing. These descending pathways are important in the maintenance of general inhibitory controls on dorsal horn neurones

either directly, or indirectly via excitatory or inhibitory interneurones at the spinal cord level. A number of pharmacological agents have been found to effect the activity of dorsal horn interneurones. Various transmitters are associated with inhibitory effects, including the opioid peptides, serotonin (5-HT), noradrenaline, dopamine and the inhibitory amino acids γ-aminobutyric acid (GABA) and glycine (see Besson and Chaouch, 1987 for review). These inhibitory neurotransmitters may be released directly by descending control systems to exert inhibitory effects (in the case of monoamines) or indirectly by the activation of spinal interneurones (Besson and Chaouch, 1987). Although of interest, the monoamines are beyond the initial scope of this study and will not be discussed further, (for review see, Besson and Chaouch, 1987).

1.8.2 Tonic Descending Inhibition

Many areas of the brain exert a potent descending inhibition on spinal somatosensory mechanisms, in particular nociceptive transmission in the spinal dorsal horn. A tonic inhibitory system was found to affect flexion reflexes in limbs and other forms of the flexion reflex, such as the tail flick reflex in the rat. The tail flick test is a nociceptive reflex, which is enhanced following spinal transection (Irwin et al., 1951). A very useful method for producing a reversible cold block of tonic descending inhibition was introduced by Brown (1971). This technique revealed that a number of different dorsal horn neurones are under tonic inhibitory control. Cold block at the thoracic level of the spinal cord enhanced the responses of spinothalamic tract (STT) cells to peripheral stimuli (Brown, 1971). Cervero (1977) found that during cold block of the cord, the majority of STT cells respond to excitation or inhibition. Tonic descending inhibitory controls affect nociceptive transmission in Lamina I cells and Lamina IV-V (Besson et al., 1975; Brown, 1971; Duggan et al., 1981), yet cells in lamina II are not subject to tonic descending inhibition (Cervero et al., 1979). The precise origin of tonic descending inhibition is unclear, although it is becoming increasingly evident that pathways originating in the medial part of the medulla and pons are likely to contribute to the descending inhibitory (and excitatory) control of spinal neurones. Labelling studies have identified that cells of the nucleus raphe magnus, pallidus and obscurus and nuclear reticularis

gigantocellularis, magnocellularis and pontis caudalis contribute to descending modulation (Willis, 1988). Cells of the lateral reticular nucleus also appear to contribute, as bilateral lesions in these areas appear to prevent such an inhibition (Foong and Duggan 1986; Morton et al., 1983).

1.8.3 Stimulus-Evoked Modulation from Supraspinal Sites

Potent descending inhibitory influences that can be elicited from the brain stem have been implicated in the modulation of pain. Several studies have highlighted that stimulation of areas of the periaquaductal grey matter (PAG) (Oliveras et al., 1974), nucleus raphe (NRM) (Guilbaud et al., 1977), and the caudal lateral reticular nucleur (LRN) (Morton et al., 1983) provide analgesia. Interestingly, stimulation of these regions of the brainstem results primarily, (but not exclusively), in the inhibition of activity evoked by noxious rather than innocuous stimuli applied to the periphery (Duggan and Griersmith, 1979; Willis et al., 1977).

1.8.4 Segmental Controls

As proposed by Melzack and Wall (1965) in the "gate control" theory, the transmission of messages at the spinal relays is under both segmental and supraspinal controls. Segmental controls are characteristically inhibitory effects produced by large-diameter fibres on the responses of spinal neurones to nociceptive stimulation (Besson and Chaouch, 1987). This has been clearly established by several groups (Brown 1971; Cervero et al., 1976). The inhibition can be produced on both nocispecific non-nociceptive neurones and can also be achieved by dorsal column stimuation (Besson and Chaouch, 1987). The "gate control" theory proposed that cells in the superficial dorsal horn operate to control the transmission of afferent volleys. This control is mediated in part, by pre-synaptic inhibition of the primary afferent fibres and is itself influenced by the activity of primary afferents; Aβ stimulation augments the inhibition, and Aδ and C fibres reduce it. Facilitation may then be exerted by the activation of fine fibres that reduce the inhibitory controls. In contrast, the activation of Aβ fibres excites the inhibitory substantia gelatinosa cells, producing an increase in inhibition of transmission. (Besson and Chaouch, 1987).

Although there has been intense controversy over this theory, neurones of the superficial dorsal horn have been implicated in segmental controls of the transmission of nociceptive messages. At least part of this control results from pre-synaptic mechanisms presumed to be acting via axoaxonic synapses where primary afferent terminals are post-synaptic (Rethelyi and Szentagothai, 1969). However, there is evidence that suggests that dendroaxonic synapses play a role in pre-synaptic inhibition (Gobel et al., 1980). From the pharmacological point of view, several substances have been implicated, notably γ-aminobutyric acid (GABA), glycine and the endogenous opioids, in the mechanisms of pre-synaptic control.

1.8.5 γ-Aminobutyric Acid (GABA)

There is considerable evidence that the inhibitory neurotransmitter GABA is present in the dorsal horn of the spinal cord, and is implicated in the control of sensory processing at the spinal cord level. Immunohistochemical studies have demonstrated the presence of GABA in approximately one third of cell bodies and axons in lamina I-III (Barber et al., 1982; Todd and McKenzie, 1989). Ionophoretically applied GABA has been shown to reverse the excitatory effect of L-glutamate in lamina II dorsal horn neurones (Curtis et al., 1959; Zieglgansberger and Sutor, 1983). Other evidence implicating a role for GABA in pre-synaptic inhibition is the depressive action on pre-synaptic inhibition of locally or intravenously applied GABA antagonists picrotoxin and bicuculline (Besson et al., 1971; Repkin et al., 1976).

1.8.6 Glycine

Glycine is an important neurotransmitter with widespread distribution throughout the spinal cord. Glycine is present in high concentrations in the grey matter of the spinal cord, particularly in the deeper laminae (III-VI) and the ventral horn (Graham et al., 1967; Todd, 1990). Ionophoretic application of glycine into the spinal cord results in a strychnine-sensitive depression of the activity of single cases in approximately 90% of neurones tested (Curtis et al., 1967; Werman et al., 1968; Zieglgansberger and Sutor, 1983; Sivilotti and Woolf, 1994). Glycine is thought to be an important modulator of SP-evoked responses, as it has been shown to be released following

microdialysis of SP in to the spinal cord in vivo (Smullin et al., 1990) and following bath application in vitro (Maehara et al., 1993). In addition glycine inhibits SP evoked biting and scratching behaviour (Beyer et al., 1989), while intrathecal application of the glycine antagonist strychnine facilitates the nociceptive flexor reflex (Silvilotti and Woolf, 1994). However, it is now commonly understood that glycine may have two opposing functions in nociceptive processing, not only its well established inhibitory action via the strychnine-sensitive glycine receptor (Pullen and Powel, 1992), but also an excitatory action via the glycine co-agonist binding site on the NMDA (N-methyl-D-aspartate) receptor (Gly_{NMDA}). It has been shown for example that the NK-1 receptor facilitation of the NMDA receptor can be blocked by ionophoretic application of Gly_{NMDA} site antagonists, whereas glycine acting via the strychnine site has inhibitory effects (Heppenstall and Fleetwood-Walker, 1997a; 1997b). The modulatory influences of glycine therefore are quite complicated, and can be exerted through several different pathways.

1.8.7 Endogenous Opioids

Various techniques have demonstrated the presence of opioid receptors on the terminals of primary afferent fibres (Wamsley, 1983, Gouarderes et al., 1999 Abbadie et al., 2001) projecting into the superficial laminae of the dorsal horn, suggesting a role for endogenous opioids in pre-synaptic control in the transmission of nociceptive information. Opioid receptors of μ, δ and κ subtypes have all been localised in the superficial dorsal horn of the spinal cord (Atweh and Kuhar, 1977; Calza et al., 2000; Dun et al., 2000; Aicher et al., 2000; Zhang et al., 2000). Opioids can act directly on the spinal cord, and ionophoretic injection of opioids inhibits spinal nociceptive processing (Duggan and North, 1983; Fleetwood-Walker et al., 1988). This inhibitory action may be pre-synaptic or may be an indirect action in response to activation of opioid-containing interneurones. Electrophysiological and behavioural experiments have shown that the powerful analgesic effects of opioids result from actions at both spinal and supraspinal sites (Ossipov et al., 2000). It has been suggested that these endogenous opioids are released in dorsal horn from opioid spinal interneurones (Basbaum and Fields, 1978; Glazer and Basbaum, 1981) and recent evidence has demonstrated that opioids may coexist with other neuropeptides

in dorsal horn interneurones (Kemp et al., 1996; Todd and Spike, 1992, Todd and Johnston,1992).

Enkephalin, dynorphin and nociceptin are families of opioid peptides which are present in the dorsal horn. Each may play a role in modulation of nociceptive inputs in acute and inflammatory pain states (Lipp et al., 1991; Caudal and Mannes, 2000; Terenius et al., 2000). However, because opioid drugs appear to be poorly effective in neuropathic pain states (Arner and Meyerson.1988) this thesis will not deal extensively with spinal opioids.

1.9 Primary Afferent Inputs in the Regulation of Dorsal Horn Neurones

The afferent connectivity and anatomical organisation of the peripheral and central systems is an important issue since it defines the projection of primary afferent nociceptors into the dorsal horn and ascending tracts, and therefore predicts the site of release of afferent transmitters. Activation of primary afferent nociceptors requires an intense mechanical, thermal or chemical stimulus at the peripheral terminal, and results in the release of chemical substances from nerve terminals in the dorsal horn to mediate the transmission of nociceptive information centrally. Synaptic transfer of information is governed by the nature and amount of the transmitter released by different primary afferents, the density and identity of post-synaptic receptors, the kinetics of receptor activation and ion channel opening and closing, and the factors responsible for the removal or breakdown of transmitter. Each of these factors is also subject to pre- and post-synaptic modulatory influences. This section will consider the pharmacology of the systems that transmit nociceptive information and the chemicals and transmitters involved in this transmission and/or modulation of information.

Afferent transmitters can be characterised by their ability to evoke excitatory post-synaptic potentials (EPSPs) on second order neurones. Single unit recording of primary afferent fibres has indicated that stimulation of different primary afferent fibres will result in two distinct populations of EPSPs which are believed to be mono-synaptic. Activation of rapidly conducting primary afferent fibres will result

in an excitation of dorsal horn neurones and the generation of a rapid and brief EPSP (Gerber and Randic, 1989a,b,). In contrast the activation of small slowly conducting primary afferent fibres results in the generation of a delayed or extended duration EPSP (Urban and Randic, 1984; King et al., 1988; Schneider and Perl, 1988; Gerber and Randic, 1989a,b; Yoshimura and Jessel, 1990). These two distinct populations of EPSPs generated by primary afferent activation may reflect the presence of different classes of afferent neurotransmitters acting on the dorsal horn neurones, and these include the excitatory amino acids and peptides respectively.

1.10 Excitatory Amino Acids (Glutamate and Aspartate)

Glutamate and aspartate are the major excitatory transmitters in the CNS and mediate fast excitatory synaptic responses (Watkins and Evans, 1981). Ionophoretic application of glutamate causes excitation of dorsal horn neurones (Curtis et al., 1959) and evidence has shown that glutamate is released from primary afferent fibres following electrical stimulation (Roberts, 1974). Glutamate and aspartate are observed in approximately 50% of dorsal root ganglion (DRG) cells (Battaglia and Rustioni, 1988; Tracey et al., 1991), and are localised in both unmyelinated dorsal root axons and Aβ fibres (Westlund et al., 1989). Glutamate is associated with synaptic vesicles throughout neurones and primary afferent terminals of the superficial dorsal horn (DeBiasi and Rustioni, 1988; Miller et al., 1988; Maxwell et al., 1990 a,b).

Several lines of evidence suggest that glutamate is involved in the transmission of nociceptive information in the dorsal horn. There are higher levels of glutamate in dorsal rather than ventral roots (Graham et al., 1967; Duggan and Johnston, 1970), and glutamate is localised in the small DRG cells associated with nociception (Salt and Hill, 1983; Battaglia and Rustioni, 1988). Glutamate is also released in the dorsal horn following noxious stimulation or peripheral inflammation (Skilling et al., 1988; Sorkin et al., 1992) and immunocytochemistry has identified glutamate in Aβ, Aδ and C fibres (DeBiasi and Rustioni, 1988). The majority of units in the dorsal horn which respond to extracellular glutamate have been identified in the superficial

laminae and are activated by C fibre volleys (Schneider and Perl, 1985; 1988). In addition, intrathecal glutamate also produces behavioural hyperalgesia and spontaneous nociceptive behaviour (Aanonsen and Wilcox, 1986; 1987).

There are several receptor subtypes through which glutamate may mediate cellular effects. The ionotropic group of receptor-linked ion channels, consists of (i) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), (ii) Kainate and (iii) N-methyl- D-aspartate (NMDA) receptors. The metabotropic glutamate receptors (mGluRs) are coupled through GTP-binding proteins to various second messenger systems.

1.10.1 AMPA Receptors

AMPA receptors are prevalent throughout the CNS (Monaghan et al., 1984) and in the spinal cord are concentrated in the superficial laminae (LI-III) where they are believed to be located on neurones post-synaptic to the primary afferent in the dorsal horn (See figure 1.3). AMPA receptors can be composed of one or any two of the GluR 1-4 subunits (Boulter et al., 1990; Nakanishi et al., 1990). Their activation leads to a potent depolarisation of dorsal horn neurones, conversely a blockade of AMPA receptors attenuates synaptic activation of dorsal horn neurones by noxious and non-noxious stimuli (Dougherty et al., 1992).

AMPA receptors are believed to mediate the fast synaptic transmission brought about by glutamate release (Jahr and Jessell, 1985; Jahr and Yoshioka, 1986; Gerber and Randic, 1989). Peripheral administration of AMPA into the glabrous skin of the rat hindpaw results in pain behaviours in rats, which can be reversed by local application of the inhibitor 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) at concentrations selective for AMPA receptors (Zhou et al., 1996). AMPA is believed to increase the response of spinal neurones to noxious mechanical stimulation (Aanonsen et al., 1990), and administration of the inhibitor (CNQX) can decrease dorsal horn neurone responses to both innocuous and noxious mechanical stimuli (Dougherty et al., 1992; Neugebauer et al., 1993), noxious thermal stimuli and Aβ and Aδ strength electrical

stimuli (Dougherty et al., 1992). Electrically evoked C fibre inputs can also be inhibited by CNQX (Blake et al., 1988; Alford and Grillner, 1990).

1.10.2 NMDA Receptors

NMDA receptors are located throughout the brain and spinal cord (particularly in the superficial dorsal horn) (Greenamyre et al., 1984; Monaghan and Cotman, 1985) where they play a key role in excitatory changes such as long term potentiation (LTP) (Collingridge et al., 1983) as well as the generation of hyperalgesia (Dougherty et al., 1992; Zhou et al., 1996). Distributional studies have shown that NMDA receptors are present on both the nerve terminals of the primary afferents as well as on membranes that are post-synaptic to the primary afferent (Liu et al., 1994). NMDA receptors are highly permeable to Ca^{2+} when activated but are readily blocked by Mg^{2+} ions in a basal state. Channel opening requires glycine, glutamate as well as the release of the magnesium block resulting from partial depolarisation of the membrane (Johnson and Ascher, 1987; Thompson et al., 1992).

Electrophysiologically, NMDA results in the potent excitation of dorsal horn nociceptors (Aanonsen et al., 1990; Dougherty and Willis, 1992; Dougherty et al., 1992). Intrathecal administration of NMDA has been shown to produce thermal hyperalgesia in the rat (Kolhekar et al., 1994). As the NMDA receptor is readily blocked by Mg^{2+} ions in a basal state, it is believed that the role of NMDA receptors in brief "physiological pain" is limited and that the NMDA receptor may play a more integral role in the development of intense or chronic pain states, where other inputs may exert the depolarisation required to release Mg^{2+} block of the channel (see section 1.13).

1.10.3 Metabotropic Glutamate Receptors (mGluRs)

The metabotropic glutamate receptors comprise eight receptors and are sub-divided into three groups, according to their amino acid homology, pharmacology and signal transduction profiles: group I (mGluRs $_{1/5}$), group II (mGluRs $_{2/3}$) and group III (mGluRs $_{4/6/7/8}$) (Masu et al., 1991; Abe et al., 1992; Nakanishi, 1992; Pin and

Duvoisin, 1995). The mGluRs, which couple via G proteins to several signal transduction pathways, regulate neuronal excitability in the CNS by indirectly modulating a variety of ion channels (for review, see Saugstad et al., 1995). Many of the mGluRs have been shown to be located in the spinal cord, where they appear to play a role in mediating nociceptive inputs in the dorsal horn of the spinal cord (Neugerbauer et al., 1994a,b; Young et al., 1994; 1995; 1997; 1998). In particular (but not exclusively) group I mGluR $_{1/5}$ receptors have been implicated in nociceptive responses (Young et al., 1994; 1995; 1997; 1998; Fisher and Coderre, 1996 a, b; Fundytus et al., 1998), and their actions may be mediated in part by protein kinase C (PKC) (Young et al., 1995), protein kinase A (PKA), and the nitric oxide activated cGMP protein kinase family (PKG) (Woolf and Costigan, 1999; Sluka, 1997; Wu et al., 1998). (see figure 1.3). Post-synaptic group I mGluRs can also modulate both AMPA and NMDA receptor-mediated currents in various neuronal populations (Neugebauer et al., 1999) and in the spinal cord (Bleakman et al., 1992; Cerne and Randic, 1992) in vitro. Similarly, activation of mGluRs can potentiate the responses of dorsal horn neurones to NMDA and AMPA receptor activation in vivo (Neugebauer et al., 1994 a, b; 1999), an effect which is mediated through group I mGluRs (Bond and Logdge, 1995; Jones and Headley, 1995).

Behavioural studies suggest that group I agonists can facilitate the effects of NMDA and/or non-NMDA receptors to produce enhanced nociceptive responses and hyperalgesia (Coderre and Melzack, 1992; Fisher and Coderre 1996 a, b; Meller et al., 1993; 1996 a, b). Metabotropic glutamate receptors undoubtedly have a role in the transmission of nociceptive information, and this role may be more substantial in a sensitised state. However, mGluRs are only one of a large number of receptors that contribute to nociceptive processing, and several other receptors have been implicated in nociception and sensitisation.

1.11 Neuropeptides

Immunohistochemical studies have demonstrated the presence of a number of neuropeptides in the primary afferent fibres and some of the key candidates for a role of somatosensory processing are listed below:

1.11.1 Substance P

The importance of substance P and NK-1 receptors in pain processing is well established (Cao et al., 1998; De Felipe et al., 1998). Substance P (SP) is a member of a family of related peptides, the tachykinins, and is considered to be an excitatory transmitter released by primary afferent neurones terminating in the dorsal horn of the spinal cord (Pernow, 1983). The anatomical distribution of SP in the rat central nervous system highlights its potential role as an important sensory neurotransmitter/neuromodulator (Quirion et al., 1983). 50% of all C fibres and 20% of Aδ fibres contain SP, and SP is present in approximately 20% of all DRG neurones specifically the small diameter neurones (Ju et al., 1987; Hokfelt et al., 1976). SP is also abundant in the spinal cord, and SP immunoreactivity is located in all laminae, particularly in LI-III, the central canal and ventral horn (Hokfelt et al., 1980; Gibson et al., 1981). About 50% of the SP in the dorsal horn is of primary afferent origin, and is located predominantly in unmyelinated primary afferent fibres (Nagy et al., 1981). It has been demonstrated that SP may be released from both the central and peripheral terminals of these neurones (Hokfelt et al., 1976). SP exerts its effects by binding to the NK-1 receptor which is densely expressed in the superficial dorsal horn of the spinal cord and to a lesser extent the deeper dorsal horn (Stucky et al., 1993; Helke et al., 1986; Naastrom et al., 1992). The tachykinin receptors are G protein coupled, and increase intracellular ca^{2+} levels.

Direct evidence of a neurotransmitter role for SP has been revealed in a number of behavioural and electrophysiological studies. Behavioural studies have demonstrated that intrathecal injection of SP in mice elicits a dose-related biting and scratching response, which are considered to reflect painful sensations (Hayes and Tyers, 1979; Hylden and Wilcox, 1981; Yashpal et al., 1982), although this analogy has been questioned (Frenk et al., 1988). Ionophoretic application of SP increases the excitability of dorsal horn neurones (Henry, 1976; Zieglgansberger and Tulloch, 1979) and selectively activates high threshold multireceptive Lamina I and II neurones (Randic and Miletic, 1977). In addition, SP enhances the responses of multireceptive and nocispecific neurones to C fibre strength stimulation (Kellstein et al., 1990). There is also evidence that the excitatory effect of SP and noxious

stimulus-evoked pain behaviour can be blocked by selective antagonists of the NK-1 receptor (Laird et al., 2001). There is now a large body of evidence implicating SP and NK-1 receptors in sensory processing in the dorsal horn.

1.11.2 Calcitonin Gene-Related Peptide (CGRP)

Among the many peptides that are located in DRG, CGRP provides one of the best examples of a neuromodulator (Levine et al., 1993) in the sense that the peptide does not exert marked excitatory effects itself on nociceptive responses, and yet dramatically potentiates the effects of other compounds, particularly SP.

CGRP like immunoreactivity has been demonstrated in many dorsal root ganglion cells including some large primary sensory neurones and particularly those with unmyelinated or small diameter myelinated primary afferent fibres (Rosenfeld et al., 1983). CGRP cells constitute about 50% of all and 70% of the small/medium sized neurones and have been shown to terminate in the dorsal horn LI, II and V (Carlton et al., 1988). CGRP is one of the most abundant and frequently occuring peptides so far encountered in sensory neurones and CGRP containing terminals in the dorsal horn appear to derive exclusively from DRG cells as dorsal rhizotomy virtually eliminates CGRP in the cord (Carlton et al., 1988). A population of CGRP containing primary afferent neurones is identical to previously described SP neurones (Gibson et al., 1984; Wiesenfeld-Hallin et al., 1984). Up to 80% of SP containing DRG neurones co-contain CGRP (Garry et al., 1989; Wiesenfeld-Hallin et al., 1984).

During nociceptive transmission CGRP is released into the superficial dorsal horn (Morton and Hutchison, 1989) implicating this peptide as a possible mediator of nociceptive transmission. Noxious thermal, mechanical or electrical stimulation has been demonstrated to evoke the release of CGRP in the superficial dorsal horn (Morton and Hutchison, 1990). Ionophoretically applied CGRP produces a slowly developing modest, but long lasting excitation of nociceptive dorsal horn neurones in vivo (Miletic and Tan, 1988), and the application of antisera to CGRP has been demonstrated to exert antinociceptive action (Kuraishi et al., 1988). Concomitant in

vitro studies have demonstrated that CGRP produces a slow depolarisation by a direct action on nociceptive dorsal horn cells (Ryu et al., 1988).

The pronociceptive role of CGRP and substance P are considered to be mediated by their respective receptors located post-synaptically at the level of the superficial dorsal horn (Garry et al., 1991; Yashpal et al., 1991 a, b, c; Levine et al., 1993). However, it is of interest to note that CGRP, in addition to its direct actions on dorsal horn neurones, may potentiate the excitatory effects of substance P through attenuating its breakdown by saturating degrading endopeptidases (Le Greves et al., 1985).

1.11.3 Somatostatin

Somatostatin has also been implicated as a neurotransmitter within the dorsal spinal cord. Somatostatin is a tetradecapeptide that in general exerts an inhibitory action (Hokfelt et al., 1975), although in electrophysiological experiments excitatory actions have been reported (Salt et al., 1982). Somatostatin is present in small neuronal cell bodies and in spinal ganglia in primary afferent fibres (Hokfelt et al., 1976) and it is generally associated with C afferent activation, possibly C-thermosensitive afferents (Wiesenfeld-Hallin, 1986). In the dorsal horn the highest concentration of somatostatin is in lamina II (Hokfelt et al., 1976) where it has been shown that somatostatin and substance P generally do not coexist (Tuchscherer and Seybold, 1985), although a small population (26%) of primary afferents do contain somatostatin and SP (Hokfelt et al., 1976; Dodd et al., 1984).

Somatostatin inhibits the firing of nociceptive specific spinal cord neurones (Randic and Miletic, 1978; Sandkuhler et al., 1990), and in vitro studies indicate that the somatostatin-induced inhibition of dorsal horn cells is associated with a hyperpolarisation of these neurones (Murase et al., 1982). The functional role for this peptide as a neurotransmitter is not very clear and it has been suggested that somatostatin may affect the release of other transmitters (MacDonald and Nowak, 1981). In addition, multiple receptor subtypes for somatostatin have now been identified. This may explain the discrepancies in the electrophysiological studies and

would suggest that the primary role for this peptide may be as a modulator rather than a transmitter.

1.11.4 Vasoactive Intestinal Polypeptide (VIP)

VIP is a neuropeptide thought to behave as an excitatory neurotransmitter within the CNS and is widely distributed in the peripheral and central nervous system (Hokfelt et al., 1980; 1982). The spinal cord contains relatively small amounts of VIP relative to other parts of the CNS. VIP is found in the dorsal horn in LI, II and V and the area around the central canal, regions where Aδ and C afferents terminate. VIP levels are generally undetectable in normal animals and there are few or no immunoreactive cell bodies found in the DRG of these animals. Sciatic nerve stimulation in the cat at intensities that activate small diameter myelinated Aδ and C afferents causes the release of VIP (Yaksh et al., 1982) and intrathecal (i.t.) VIP causes an increase in spinal cord excitability primarily to cutaneous thermal stimuli in decerebrate animals. The levels of VIP dramatically increase following peripheral axotomy (Noguchi et al., 1989) and CCI (Nahin et al., 1994) and so it is possible that its transmitter role becomes more important following nerve injury (section 1.18.5). VIP and the related peptide Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) exert their effects through the VPAC and PAC receptor family which will be discussed in great detail in chapter 3.

1.11.5 Neuropeptide Y (NPY)

NPY is a peptide with 36 amino acids, which is widely distributed in the mammalian central and peripheral nervous systems (Tatemoto et al., 1982; Hokfelt et al., 1983 a, b). NPY is found in all levels of the spinal cord particularly in the dorsal horn (Gibson et al., 1984) and is most abundant in the lumbosacral regions. Rhizotomy, or application of capsaicin do not result in changes in the levels of NPY and so the source of NPY containing fibres in the normal dorsal horn is thought to be the CNS, with immunoreactivity not normally detectable in the DRG (Gibson et al., 1984). NPY immunoreactive fibres are concentrated in LI and II and NPY binding sites in lamina I and II are reduced in numbers after depletion of primary afferent fibres by

rhizotomy or capsaicin treatment. This suggests that NPY is present on NPY sensitive unmyelinated fibres. The overall distribution of NPY is similar to VIP and CCK and NPY has been shown to co-exist with somatostatin in primary afferent fibres and nerve terminals (Gibson et al., 1984).

NPY is an inhibitory neuropeptide, although a variety of receptors have now been identified which may serve to mediate distinct actions. Microinjection of NPY in the dorsal horn has been shown to evoke a stimulus induced decrease in SP release from primary afferents (Duggan et al., 1991), suggesting that NPY could cause pre-synaptic inhibition of primary afferent transmitter release. In addition, NPY administered intrathecally can produce analgesia in conscious rats (Holets et al., 1988). The majority of NPY containing neurones in the dorsal horn have been shown to be GABAergic (Rowan et al., 1993), and NPY may therefore be acting in conjunction with GABA in order to produce pre-synaptic inhibition. Following neuropathic injury NPY expression in DRG cells, especially those with large diameter axons is markedly increased (McMahon and Priestley, 1995), so the role of NPY may become considerably more significant in nerve injury.

1.11.6 Cholecystokinin (CCK)

The neuropeptide CCK is found within the CNS in a number of different mammalian species (Yaksh et al., 1988). CCK is expressed primarily in the small diameter DRG, as well as in peripheral nerves and the superficial layers of the dorsal horn (Fuji et al., 1983; 1985). However like VIP, the levels of CCK are relatively low in the dorsal horn of normal animals (Noguchi et al., 1993) and its role is believed to be more important following nerve injury when it is substantially upregulated (Hokfelt et al., 1994; Noguchi et al., 1989).

1.11.7 Galanin

Galanin receptors are widely distributed throughout the CNS particularly in the superficial dorsal horn (Wiesenfeld-Hallin et al., 1992; Zhang et al., 1995). It is normally present in around 23% of small to medium diameter DRG neurones, where

it co-exists with several other neuropeptide transmitters including CGRP and SP (Ju et al., 1987; Zhang et al., 1993; 1995). Galanin levels have been shown to decrease in the superficial laminae (LI-II) by neonatal capsaicin treatment (Skofitsch and Jacobowitz, 1985), suggesting that spinal galanin arises substantially from primary afferent C fibres. The role of galanin within the CNS is not very clear, as functional studies have provided variable results depending on the preparation and dose used, and the effect studied. In rats with an intact nerve, intrathecal galanin produces brief facilitatory effects at low doses, which become inhibitory as the dose increases, such that at very high doses galanin exerts a purely inhibitory effect (Wiesenfeld-Hallin et al., 1988; 1989). Again, multiple receptor subtypes probably contribute to the complex profile of effects. Galanin may be an important modulator of excitatory neuropeptide action, as pre-administration of galanin intrathecally has been shown to antagonise the excitatory effects of SP and CGRP on the flexor reflex (Wiesenfeld-Hallin et al.; 1990, 1991; Xu et al., 1989).

1.12 Central Integration of Afferent Signals in Prolonged / Repetitive Inputs

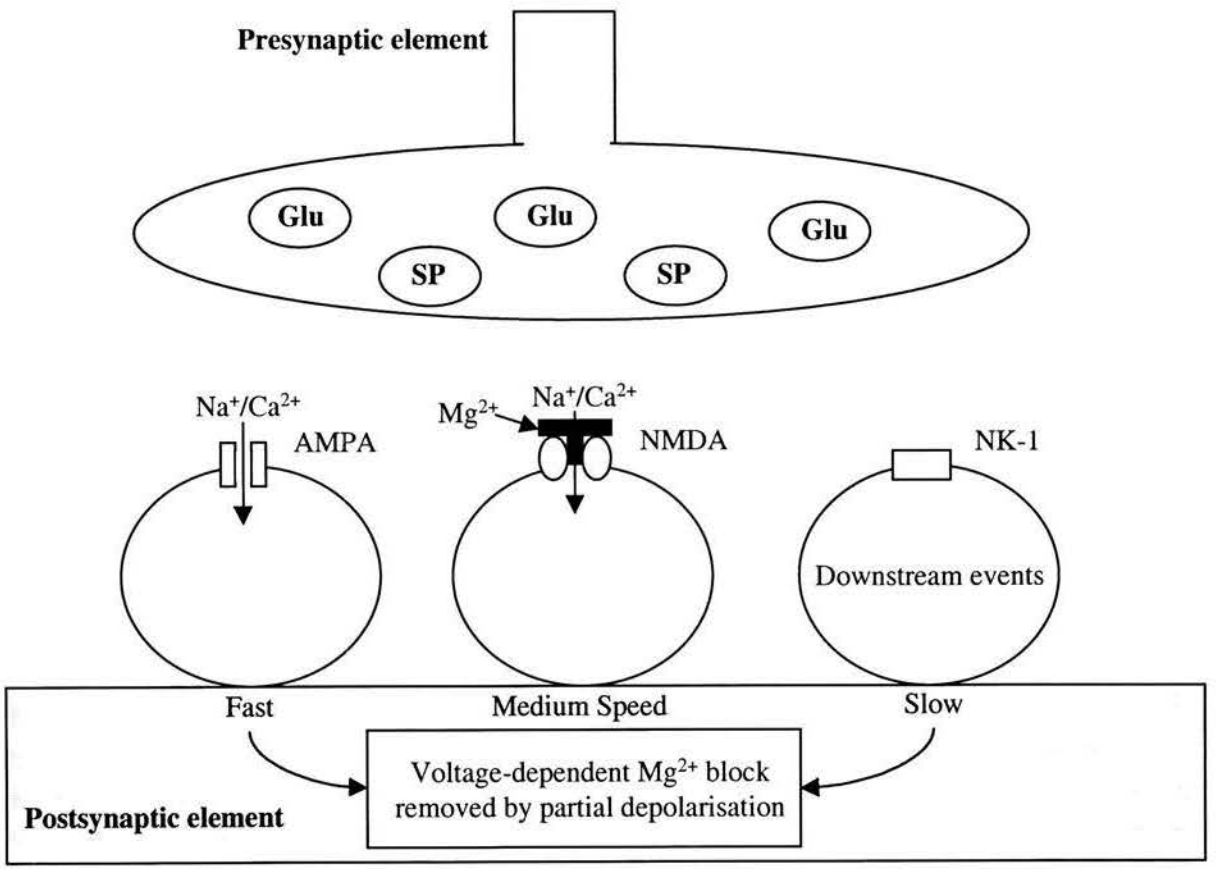
A vast number of different neurotransmitters / neuromodulators thus appear to be able to influence the nociceptive processing systems within the spinal dorsal horn, and it seems likely that there is no sole mediator of nociceptive transmission. Instead it seems more likely that a variety of neurotransmitters interact within the CNS to mediate / regulate the transmission of nociceptive (and non-nociceptive) information.

It seems almost inevitable that the influences of these agents will be superimposed on those of the excitatory amino acids released from afferents (Figure 1.3). The excitatory amino acids act at NMDA, AMPA, kainate and mGluR receptors (Price et al., 1994). It appears that the AMPA receptor may be substantially involved in mediating responses in the acute "physiological" processing of sensory information. However, with prolonged release of glutamate or activation of neurokinin receptors, a secondary process occurs which appears to be crucial in the development of abnormal responses to further sensory stimuli. Glutamate and aspartate stimulate the influx of Ca^{2+} through NMDA receptor-operated channels (MacDermott et al., 1986),

as well as increasing Ca^{2+} influx through voltage gated Ca^{2+} channels associated with AMPA/kainate receptors (Murphy and Miller, 1989), and causing the mobilisation of Ca^{2+} from internal stores after the activation of metabotropic glutamate receptors (Berridge and Galione, 1988). A sustained activation of AMPA (or alternatively perhaps neurokinin or metabotropic glutamate) receptors may cause the partial depolarisation necessary to "prime" NMDA receptors and the concomitant elevation of Ca^{2+} may further enhance phosphorylation-dependent modifications of NMDA receptors as well as of voltage-dependent ion channels (Dray et al., 1994). Together, these prolonged changes can result in the development of NMDA receptor-dependent central sensitisation, as for example in "wind up" (a facilitation of dorsal horn neurone responses caused by repetitive afferent stimulation), and expansion of the peripheral receptive fields (Davies and Lodge, 1987), and it appears that the NMDA receptor is needed for these responses (Dickenson and Sullivan, 1987). This supports the idea that NMDA receptors become more involved in the transmission of nociceptive information, particularly if the system has undergone some form of sensitisation. In the highly sensitised state resulting from peripheral nerve injury NMDA appears to play a major facilitatory role.

Figure 1.3. Post-Synaptic Integration of Signals in Gating the Role of NMDA Receptors

Afferent transmitters / neuromodulators act on receptors and ion channels in the dorsal horn to elicit Na^{2+} / Ca^{2+} entry and / or activate protein kinases that phosphorylate membrane bound NMDA and AMPA receptors and alter their functional properties. These processes then act to prime the NMDA receptor by removal of the Mg^{2+} channel block and allow further Na^{+} and Ca^{2+} influx to enhance membrane excitability and Ca^{2+}-dependent intracellular responses, which are likely to play a key role in central sensitisation.


Presynaptic element
Glu
Glu
Glu
SP
SP
Na+/Ca2+
AMPA
Mg2+
Na+/Ca2+
NMDA
NK-1
Downstream events
Fast
Medium Speed
Slow
Voltage-dependent Mg2+ block
removed by partial depolarisation
Postsynaptic element

1.13 "Wind-Up" and Central Sensitisation

A continued barrage of nociceptive inputs to the CNS results in changes to the response properties of dorsal horn neurones (Chi et al., 1993). It has been demonstrated that repetitive stimulation of C-afferent fibres not only activates dorsal horn neurones, but neuronal activity also progressively increases throughout the duration of the stimulus (Mendell, 1966). Wind up is frequency dependent and relies essentially only on the features of the C fibre afferent inputs and not the post-synaptic cell (Davies and Lodge, 1987). Activation of AMPA, mGluR and NK-1 receptors on dorsal horn neurones will produce slow synaptic potentials in response to C fibre primary afferent activity and result in the progressive depolarisation, which results in the removal of the Mg^{2+} block from the NMDA receptor. Wind-up is dependent on activation of the NMDA receptor (Dickenson and Sullivan, 1987; Woolf and Thompson, 1991), and studies have demonstrated that wind-up can be mimicked by the application of L-glutamate or NMDA (Gerber and Randic, 1989 a, b; King et al., 1985. These studies, together with electrophysiological studies, using the electrically evoked wind-up model have consistently observed the ability of the NMDA receptor antagonists to decrease or abolish ongoing C fibre activity (Davies and Lodge, 1987; Dickenson and Sullivan, 1987; 1990; Thompson et al., 1990). Experiments in which electrically-evoked synaptic responses have been broken down into separate (early and late) components (Gerber and Randic 1989 a, 1989b; Dickenson and Sullivan, 1990) have therefore suggested that NMDA receptors play a selective role in high threshold C fibre inputs to the dorsal horn.

The activity-dependent alterations in dorsal horn neurone responsiveness brought about during wind-up are thought to be part of the phenomenon of "central sensitisation". Low frequency repetitive nociceptor input or peripheral tissue damage leads to functional changes in the processing of nociceptive information in the dorsal horn (Woolf, 1984). These changes include: increased responsiveness to suprathreshold inputs, expansion of receptive field size so that neurones are activated by stimuli normally outside the receptive field area, reduction in thresholds for activation such as an innocuous Aβ fibre input resulting in an exaggerated response ("allodynia"), and prolonged afterdischarge (Woolf, 1983; Woolf and Wall, 1986;

Dickenson and Sullivan 1987; Simone et al., 1989; Haley et al., 1990; Woolf and King, 1990; Coderre and Melzac, 1992; Dougherty and Willis, 1992; Woolf and Doubell, 1994). These modifications collectively comprise central sensitisation. An additional manifestation of central sensitisation is secondary hyperalgesia, whereby an area outside the site of injury experiences increased sensitivity to noxious stimuli. Evidence for a central component includes a demonstration that local anaesthetic administration in the primary area prevents capsaicin-induced secondary hyperalgesia to mechanical stimuli only if administered before the capsaicin injection (LaMotte et al., 1991). NMDA is key in the development of central sensitisation, and in behavioural studies, secondary hyperalgesia, due to noxious thermal injury can be prevented by pre-treatment with an NMDA antagonist (Coderre and Melzack, 1991). The central sensitisation and consequently altered sensory processing following nerve injury may additionally lead to structural changes (see section 1.20).

1.14 Post-synaptic Effects of Neurotransmitters/Neuromodulators

Excitatory amino acids and neuropeptides appear to contribute to nociception by direct neurotransmitter actions or neuromodulatory mechanisms on post-synaptic ion channels / receptors. However, the prolonged changes occuring due to a sustained noxious input cannot be fully attributed to such rapid effects. Instead, they are likely to be mediated by the subsequent intracellular events triggered by the activation of these receptors.

1.14.1 Intracellular Second Messengers Involved in Nociception

Evidence suggests that there is a contribution of intracellular Ca^{2+}, second messenger systems and protein kinases that phosphorylate membrane bound proteins (Nestler and Greengard, 1983), leading to the development of noxious stimulus-induced neuroplasticity. In addition to increases in intracellular Ca^{2+}, excitatory amino acid and neuropeptide receptor activation results in activation of various intracellular second messengers. The influx of Ca^{2+} through NMDA receptor channels activates nitric oxide synthase (NOS) which generates NO from free L-arginine (Garthwaite et al., 1988), which in turn activates soluble guanylate cyclase and increases cGMP

(Southam et al., 1991). The Ca^{2+} influx also activates neuronal phospholipase A_2 (PLA_2), which triggers the production of arachidonic acid (Dumuis et al., 1988; 1990), which is metabolised into various eicosanoids. Activity of metabotropic glutamate receptors triggers an increase in phosphoinositide metabolism, resulting in the production of inositol triphosphates (Sladeczek et al., 1985). This leads to a release of Ca^{2+} from intracellular stores (Murphy and Miller, 1988), as well as production of diacylglycerol which stimulates the translocation and activation of PKC (Manzoni et al., 1990). Activation of calmodulin / Ca^{2+}-dependent kinase II (CamKII) by Ca^{2+} and calmodulin downstream of inositol-1,4,5-triphosphate (IP_3) metabolism (Erondu and Kennedy, 1985) is widely believed to be involved in the downstream influences and regulation of glutamate transmission (Wu et al., 1986; McGlade-McCulloh et al., 1993; Watanabe and Onozuka, 1994). In addition, activation of the G protein coupled neuropeptide receptors (such as the CGRP, VIP and PACAP receptors) results in the activation of adenylate cyclase and an increase in cAMP production, which in turn triggers the activation of cAMP-dependent protein kinase (PKA) (Menikoth et al., 1993) (as described in chapter 4).

Persistent nociception as a result of tissue injury for example, may result in the repetitive afferent barrage that bring about altered peripheral and central sensory processing. However, once the injury is healed, the central sensitisation will abate and the abnormal nociceptive sensations will resolve. In contrast, pain resulting from nerve injury can lead to long term structural and functional changes in both the peripheral and central nervous system that bring about a state of altered sensory processing and the establishment of a chronic pain state.

1.15 Neuropathic Pain

Damage to a somatic sensory nerve can be expected to cause somatic sensory loss. In many patients, however, negative symptoms such as numbness are joined by positive sensations, involving in almost all cases some sort of false sensation of pain. The experience can range from mild dysesthesia (an unpleasant and abnormal pain sensation) (Merskey and Watson, 1979) to unbearable pain. Indeed, some patients are unable to work, walk, or sleep, and the contact of clothing with the skin is

experienced as an unbearable burning. Neuropathic pain usually develops into a chronic state and fails to respond to standard analgesic interventions. Morphine can give a degree of relief, but only at doses impractical for what may be a lifelong regime.

Neuropathic pain may develop whenever nerves are damaged by trauma, diseases such as diabetes, herpes zoster, cancer or chemical injury. It may also develop after amputation. The resultant nerve damage leads to pathological changes both peripherally and centrally and leads to painful neuropathic symptoms which are characterised by several different types of pain sensation:

- Spontaneous pain: this may be a continuous superficial burning pain and / or deep aching pain, or episodic attacks.
- Hyperalgesia: increased sensitivity to noxious stimulation.
- Allodynia: pain in response to a normally innocuous stimulus.

The duration of these abnormal pain states can range from months to decades (Scadding, 1984), as the pathological conditions often persist long after healing of the damaged peripheral tissue or nerve.

1.16 Changes in Primary Afferent Responsiveness Following Nerve Injury

It is possible that the spontaneous activity of injured primary afferents may contribute to the induction and maintenance of the allodynic and hyperalgesic states which follow nerve injury, as well as contributing to the abnormal behaviours thought to be indicative of spontaneous pain. When a peripheral nerve is sectioned, resources are mobilised for regenerative growth and the axons in the central end may undergo sprouting (Devor and Wall, 1976). Successful regeneration restores functional contact between the afferent and its target tissue and ideally re-establishes normal sensation. However, if local conditions prevent regenerative growth and the sprouts fail to reach the distal part of the cut nerve they form a tangled mass called a neuroma (Cajal, 1928). Electrophysiological recordings from neuromas have shown that many trapped afferent endings fire spontaneously. Moreover, they become

directly sensitive to mechanical, thermal and chemical stimulation (Wall and Gutnick, 1974; Devor et al., 1989). The resulting abnormal firing patterns have been termed ectopic discharges and chemicals that increase ectopic discharges (for example; catecholamines and inflammatory mediators) from neuromas have been shown to increase spontaneous pain in humans (Chabal et al., 1989). This abnormal activity appears to be principally mediated by Aβ and Aδ fibres (Kajander and Bennett, 1992; Kajander et al., 1992). Following CCI, ectopic activity continues for several weeks post-injury and ectopic discharges from C fibres begin slightly later than A fibres, from approximately day 10 onwards (Xie and Xiao, 1990), which corresponds with the maximal behavioural changes observed in the CCI model (Attal et al., 1990; Bennett and Xie, 1988). These findings suggest that alterations in the firing properties of peripheral nerves following nerve injury can contribute to the altered processing of nociception and ultimately an increase in the sensitivity of peripheral nerves, particularly the large diameter myelinated Aβ fibres. However, it is apparent that altered central nervous system processing also plays a key role.

1.17 Altered Central Nervous System Processing Following Peripheral Nerve Injury

The abnormal pain-related behaviours result in part from (peripheral sensitisation). And this phenomenon probably makes an important contribution to causing altered central nervous system processing (central sensitisation) and this central sensitisation likely is maintained both by ongoing peripheral inputs as well as mechanisms within the spinal dorsal horn itself. Persistent activation of spinal NMDA receptors can induce a local state of facilitated processing secondary to small afferent stimulation that leads to a increased receptive field size and a increased responsiveness to low and high threshold stimulation ("Wind-up"), (Mendell and Wall, 1965) (section 1.13). NMDA antagonists have also been shown to inhibit the responses of dorsal horn neurones induced by prolonged chemical nociception (Haley et al., 1990) and joint inflammation (Neugebauer et al., 1993). So it appears that the activation of NMDA receptors contribute greatly to prolonged states of nociception, even when these arise from different origins.

In addition to the depolarisation-induced relief of Mg^{2+} block, further facilitation of NMDA receptor function may arise from phosphorylation-dependent modification of the NMDA receptor itself (Chen et al., 1995). Maintenance of the sensitised state may be importantly through the persistence of these post-translational modifications of the NMDA receptor. This has important clinical implications as NMDA and NK-1 antagonists may prevent the establishment of central sensitisation but only NMDA antagonists can reverse established central sensitisation (Woolf and Thompson 1991; Ma and Woolf, 1995; Traub 1996).

The past decade has seen great progress both in understanding the causes of neuropathic pain and in finding new drugs that promise great benefit. The work has been advanced by a series of discoveries, beginning with a number of animal models that closely mimic neuropathic pain, as seen clinically. These models provide the means with which to investigate some of the mechanisms behind the hyperalgesia and allodynia induced by nerve injury, and therefore have greatly improved the understanding of the neural mechanisms underlying neuropathic pain states. Several models now exist using a partial nerve injury, which allows some correlation of the central changes which occur following nerve injury, with the severity of pain-related behaviour. A variety of tests can be performed on the affected, ipsilateral hind limb, which measure alterations in the characteristic reflex withdrawal responses from thermal, mechanical and chemical stimuli. The model of choice for this study is the chronic constriction injury (CCI) model first described by Bennett and Xie (1988).

1.18 The Chronic Constriction Injury Model (CCI)

This injury is produced by tying four chromic catgut ligatures loosely around the sciatic nerve. These ligatures are tied so as to retard, but not stop, blood flow in the epineural vessels. The resultant inflammatory response causes constriction of the sciatic nerve and within one week post-operatively rats exhibit altered spontaneous behaviours indicative of neuropathic pain. Ipsilateral to nerve injury, the affected hindpaw exhibits heat hyperalgesia, cold and mechanical allodynia (Bennett and Xie, 1988; Attal et al., 1990). In addition, Attal et al., (1990) reported that rats with CCI held the injured hindpaw in various abnormal positions, exhibited guarding

behaviour with the paw generally raised and the toes ventroflexed, which, they suggested indicated the presence of spontaneous pain. These behavioural changes have been shown to manifest around 36 hours post-operatively and the severity of these changes peak at around 2 weeks post-operatively, lasting for up to 3 months (Bennett and Xie, 1988). An important feature of the CCI model is that the ligatures do not completely sever the nerve so that the continuity of many of the axons within the sciatic nerve is maintained.

Many CCI rats exhibit additional abnormalities, reminiscent of phenomena seen in human causalgia (burning pain after nerve injury) and reflex sympathetic dystrophy (RSD; post-injury pain accompanied by sympathetic manifestations such as sweating pallor or redness). Skin temperature variability between ipsilateral and contralateral paws is common following CCI (Wakisaka et al., 1991). Also, the claws on the ipsilateral hindpaw grow abnormally thick, long and curved (Wakisaka et al., 1991). Equivalent changes are also seen in human causalgia and RSD patients.

This model has greatly facilitated experiments probing the pathophysiology of neuropathic pain, and is the model of choice for this study as it is well established and allows the comparison of present results with previous findings. In addition, the model is relatively simple and safe to set up and the incidence of autotomy and morbidity is low and the affective hindlimb is easily accessed.

1.18.1 Plasticity Following Nerve Injury

Following CCI, characteristic and abnormal behavioural manifestations develop: namely, hyperalgesia, cold and mechanical allodynia as well as behavioural signs of spontaneous pain (Bennett and Xie, 1988; Attal et al., 1990). There is evidence that several aspects of the nerve injury may contribute to the development of these abnormal pain states. Clatworthy et al., (1995) demonstrated that the local inflammatory response that follows CCI may contribute in part to the development of hyperalgesia and guarding behaviour in rats. Injection of the anti-inflammatory agent dexamethasone, decreased the inflammatory response induced by ligation of the sciatic nerve with chromic ligatures and subsequently blocked the guarding

behaviour and thermal hyperalgesia (Clatworthy et al., 1995). Concomitant data demonstrated that application of the pro-inflammatory agent Complete Freund's Adjuvant (CFA) to the site of injury resulted in the augmentation of these abnormal behaviours (Clatworthy et al., 1995). In addition, decreased bloodflow at the site of injury caused by the ligatures and accentuated by the resultant inflammation and constriction of the sciatic nerve is evident and is believed to contribute to thermal hyperalgesia (Myers et al., 1993).

Recent work by Kajander et al., (1996) has demonstrated that the suture material used to constrict the sciatic nerve itself is important in the development of abnormal behavioural responses. Comparison of chromic cat gut, plain gut, and polyglactin sutures revealed significant variation in the position in which the rats held there affected hind paws with the effects on paw position much greater in rats with chromic gut ligatures (Kajander et al., 1996). Since all types of suture succeeded in producing a degree of abnormal behavioural responses, the physical constriction of the nerve is likely to play an important part in their development. However, these results also suggest that the chemical constituents of the chromic gut sutures may also have an effect, and further evidence by Maves et al., (1995) demonstrated that providing an acidic environment around the sciatic nerve for 7 days will result in the development of thermal hyperalgesia.

1.18.2 Morphological Changes Following CCI

The rat sciatic nerve is associated with spinal segments L4-L6 and contains fibres of sensory, motor and sympathetic origin. At the mid-thigh level (site of ligation), the sciatic nerve comprises approximately 27, 000 axons of which 6% are motor axons, 23% are myelinated sensory axons, 48% are unmyelinated sensory axons while 23% represent unmyelinated sympathetic axons (Schmalbruch, 1986).

There are many morphological changes in the fibre composition of the sciatic nerve that occur following CCI, and since many axons within the nerve are left in continuity, the rat's responses to stimulating the partial denervated limb can be tested.

There are two main stages involved in the pathological changes of the sciatic nerve following CCI (Coggeshall et al., 1993). The first is an early degenerative stage, which is thought to be a direct result of the inflammation which occurs following nerve ligation, and constricts the axons. Light microscopic analysis has indicated that the ligatures predominantly affect large myelinated Aβ fibres distal to the injury (Gautron et al., 1990). These changes were confirmed in electrophysiological studies of single fibres in the distal segment of the ligated sciatic nerve. Almost no rapidly conducting fibres could be detected and a complete loss of large myelinated fibres distal to the site of nerve ligation was observed at the peak of neuropathy. Based on these studies it was suggested that the loss of large fibres underlies the sensory changes observed in this model, possibly via a loss of central inhibitory control of spinal cord nociresponsive neurones (Gautron et al., 1990; Coggeshall et al., 1993). It has also been demonstrated that large myelinated fibres are affected proximal to the site of the ligature, albeit much less so, and abnormalities have been detected in the Aδ range (Gautron et al., 1990). Basbaum et al. (1991) demonstrated considerable changes in the unmyelinated fibre spectrum following CCI, and there was a 34% and 71% decrease observed in the distal compared to proximal nerve at the peak of neuropathy. Large clusters of unmyelinated axons which usually occur in the sciatic nerve were absent distally at day 14 post-operatively and generally they appeared singly or in loose abnormal bundles.

The second stage of pathological change is the regenerative phase. This generally occurs from day 28 post-operatively onwards, when the sutures have been reabsorbed and the swelling has subsided (Coggeshall et al., 1993). This coincides with fibre regeneration and the recovery of axonal numbers, and probably accounts for the eventual recovery of normal sensation within the affected limb (Guilbaud et al., 1993).

Behavioural studies investigating the time course of degeneration in relation to behavioural abnormalities have revealed that the hyperalgesia following CCI is generally maximal at day 10-14 post-operatively (Bennett and Xie, 1988; Attal et al.,

1990). This parallels the onset of fibre loss of large diameter myelinated fibres (Gautron et al., 1990; Coggeshall et al., 1993).

The damage to nerve fibres following CCI does appear to be important as the development of abnormal behaviours appears to coincide with the degeneration and regeneration of A-fibres. However, there does not appear to be a strong and direct link between the development and subsequent recovery of abnormal behaviours following nerve injury and the time course of fibre loss. At week 15 post-operatively when behavioural signs have generally dissipated, large diameter myelinated fibres are still absent (Guilbaud et al., 1993). It does appear likely that the degeneration of nerve fibres observed following CCI is important but there are additional factors contributing to the overall development of neuropathic behaviours.

1.18.3 Afferent Neuropeptide Plasticity Following Nerve Injury.

Following nerve injury, long-lasting changes in the expression of neuropeptides and their receptors in primary afferent neurones are observed (Figure 1.4). In addition, changes in the peripheral and central transport of these substances results in marked plasticity within the spinal cord and the changes observed are assumed to represent adaptive responses to limit the consequences of the inflicted damage. The two main excitatory peptides SP and CGRP and the inhibitory neuropeptide SOM appear to be downregulated in response to the nerve injury.

1.18.4 Substance P, CGRP and SOM

Following axotomy there is a dramatic decrease in SP mRNA in the small to medium diameter DRG cells accompanied by a parallel decrease in SP immunoreactivity in the dorsal horn (Barbut et al., 1981; Jessel et al., 1979; Noguchi et al., 1993; Shehab et.al, 1986). A similar decrease in the production of SP in primary afferent fibres is also observed following CCI (Cameron et al., 1991; 1997; Nahin et al., 1994) with a significant decrease in the density of SP immunoreactive fibres in the ipsilateral spinal cord (Kajander and Xu, 1995). Concomitantly CGRP immunoreactivity is decreased following CCI (Nahin et al., 1994) and CGRP mRNAs have been shown

to fall to half their normally abundant levels in the DRG. Peripheral axotomy results in a marked decrease in the expression of CGRP in primary afferent neurone somata, which is maximal at day 7-14 post-operatively (Noguchi et al., 1989; 1990; 1993; Shehab et al., 1986). A similar finding has been observed following CCI (Nahin et al., 1994). However, decreases in the immunoreactivity of this peptide in the dorsal horn are not observed until about day 60 after nerve injury (Kajander and Xu, 1995). Sciatic nerve section is associated with a decrease in the levels of somatostatin mRNA in DRG neurones (Noguchi et al., 1989) and a corresponding decrease in the levels of peptide immunoreactivity in terminals in the dorsal horn (Barbut et al., 1981; Shehab et al., 1986; Villar et al., 1989; Zhang et al., 1993).

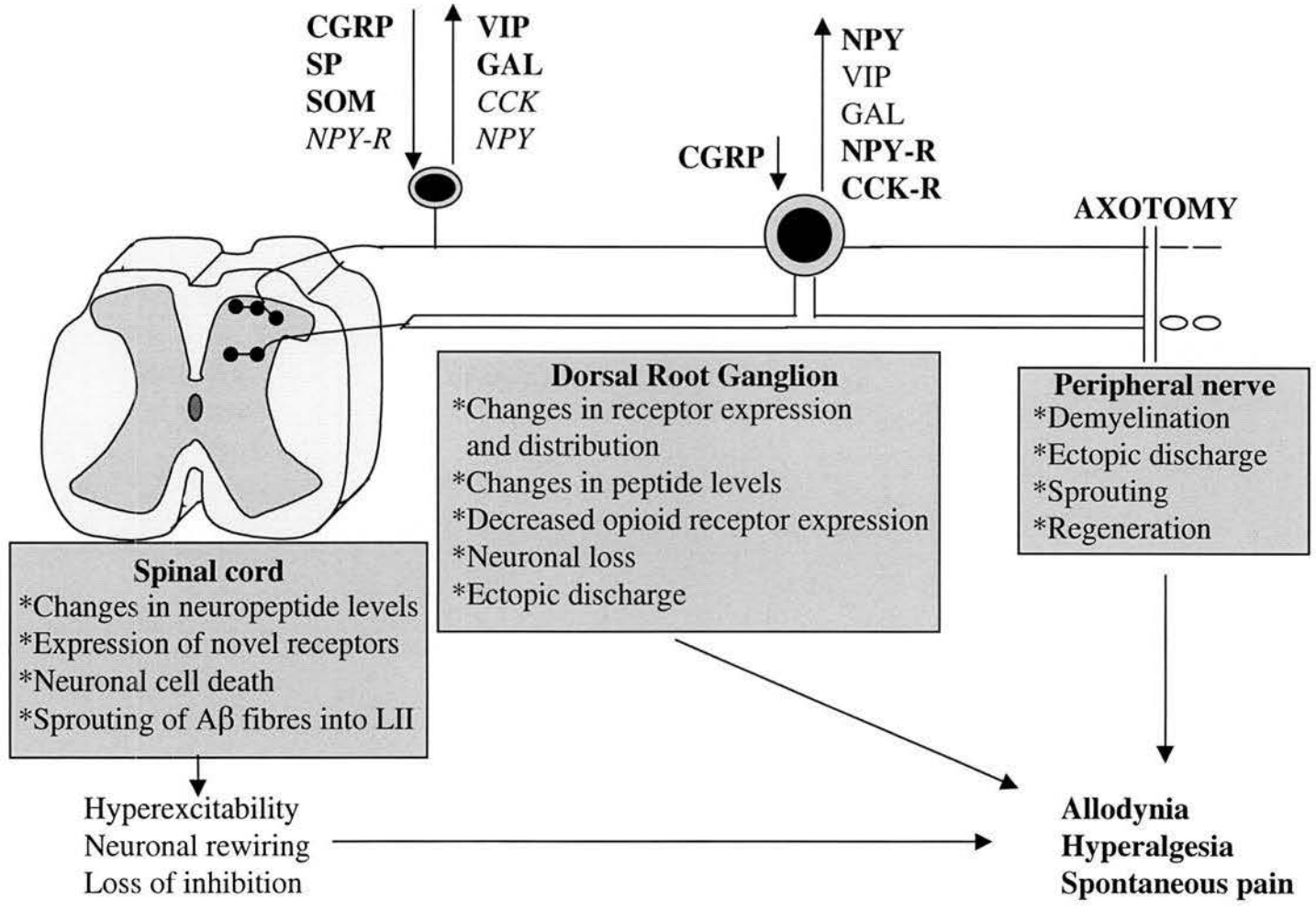
1.18.5 VIP, Galanin, NPY and CCK

In contrast to SP, CGRP and SOM, a different response pattern has been observed for a group of peptides almost undetectable under normal circumstances. Thus, following nerve injury there is a marked increase in the levels of the neuropeptides VIP, galanin, NPY and CCK (Figure 1.4). The levels of VIP dramatically increase following peripheral axotomy (Noguchi et al., 1989) and CCI (Nahin et al., 1994) from apparently undetectable levels in the dorsal horn (Knyihar-Csillik et al., 1993) and primary afferent neurones (Fuji et al., 1985) of normal animals. In double labelling studies, it was demonstrated that VIP expression only occurs in cells whose axons are cut (Shehab et al., 1986). This suggests that sensory neurones may express VIP as a direct consequence of nerve injury. VIP has a predominantly excitatory role (section 1.11.4), and it has been suggested that VIP takes over the role of SP as a primary neurotransmitter within the CNS of nerve injured animals. Peripheral nerve injury also leads to an increase in the neuropeptide galanin. Galanin mRNA increases within DRG cells within 24 hours, as well as in the ipsilateral dorsal horn (Ma and Bisby, 1997; Nahin et al., 1994; Romualdi et al., 1990; Zhang et al., 1995). The inhibitory role of galanin is more pronounced following nerve injury (Wiesenfeld-Hallin and Xu, 1996), and correspondingly the effect of the galanin antagonist M-35 on CS-induced reflex facilitation is more pronounced following nerve injury. This suggests that galanin's functional role becomes more important following nerve injury. The increase in VIP and galanin seem to occur mainly in

small and medium sized neurones, often in the same neurones and especially in those that normally contain SP and CGRP (Hokfelt et al., 1994). The level of NPY is dramatically upregulated following axotomy, mostly in large DRG neurones, that is, a population of neurones believed to be almost totally devoid of these neuropeptides (Hokfelt et al., 1994). In normal animals NPY positive fibres are present in the superficial laminae of the dorsal horn, and all evidence indicates that these fibres solely arise from local cell populations in the dorsal horn. However, following axotomy there is also a dense innervation into LIII and IV suggesting an upregulation and a presence in large calibre fibres thought to be of afferent origin (Wakisaka et al., 1991, 1992; Zhang et al., 1993). CCK, which is normally present in low amounts in DRG (Fuji et al., 1985; Ju et al., 1987) is dramatically upregulated following peripheral nerve injury at the mRNA level in primary afferent neurones (Villar et al., 1989). The role of CCK in neuropathy is not clear (See Wiesenfeld-Hallin and Xu, 1996) but the analgesic effect of morphine and β-endorphin appear to decrease as a result of its presence (Faris et al., 1983). In addition, antagonists of the CCK-B receptor (which is the prevalent form of CCK receptor within the rat spinal cord) potentiate opioid analgesia and attenuate the development of morphine tolerance (Baber et al., 1989; Dourish et al., 1990; Wiesenfeld-Hallin et al., 1990). The upregulation of CCK following nerve injury may in part help to explain the decreased efficacy of opioid analgesia prevalent in neuropathic patients.

Figure 1.4 Schematic of the Principal Changes at the Spinal Level Following Peripheral Nerve Injury

Small and large primary sensory neurone in a DRG send a central branch to the dorsal horn of the spinal cord and with a peripheral branch that has been sectioned (axotomy). The changes occurring in levels of peptides and of both peptide and peptide receptor mRNAs are indicated by arrows. Thus in small diameter, unmyelinated DRG neurones CGRP, SP and SOM are decreased as well as NPY receptor mRNA. In contrast, levels of VIP, GAL are markedly increased, with modest increases in CCK and NPY. In large diameter DRG cells there is a prominent increase in the levels of NPY and the NPY receptor, along with smaller increases in VIP and GAL, while the expression of CGRP decreases.


CGRP
SP
SOM
NPY-R
VIP
GAL
CCK
NPY
CGRP
NPY
VIP
GAL
NPY-R
CCK-R
AXOTOMY
Dorsal Root Ganglion
*Changes in receptor expression and distribution
*Changes in peptide levels
*Decreased opioid receptor expression
*Neuronal loss
*Ectopic discharge
Peripheral nerve
*Demyelination
*Ectopic discharge
*Sprouting
*Regeneration
Spinal cord
*Changes in neuropeptide levels
*Expression of novel receptors
*Neuronal cell death
*Sprouting of Aβ fibres into LII
Hyperexcitability
Neuronal rewiring
Loss of inhibition
Allodynia
Hyperalgesia
Spontaneous pain

1.19 Intracellular Mechanisms in Spinal Nociceptive Processing

1.19.1 Downstream Signal Transduction

Several studies have aimed to elucidate the intracellular mechanism(s) by which nociceptive information is transmitted in dorsal horn neurones. There are undoubtedly multiple second messenger pathways involved in this process, many of which are beyond the scope of this study. The second messenger pathway that involves the activation of the neuropeptide receptor $VPAC_2$ and the associated signal transduction cascade involving the formation of adenylate cyclase and subsequent activation of PKA was investigated in the current experiments and so will be addressed in detail in chapters 3 and 4

1.19.2 Molecular Mechanisms of Nociception

Beyond the initial effects of second messenger activation, many longer-term consequences may also be initiated. For example, in addition to altering membrane permeability, a number of signalling events, including increases in intracellular Ca^{2+} and the activation of downstream signal transduction enzymes such as PKA, converge on the phosphorylation of Ser-133 residue in the cAMP response element-binding protein (CREB). CREB is an important downstream effector of the cAMP dependent signalling pathway and positively regulates gene expression in response to changes in cAMP and Ca^{2+} dependent signalling pathways (Meyer and Habener, 1993; Ghosh and Greenberg, 1995) via a conserved cAMP response element (CRE) (Montiminy et al., 1986; Comb et al., 1986). The increased expression of immediate-early genes (IEG) such as c-fos (Morgan and Curran, 1991) generates protein products (e.g. Fos) which act as third messengers that are believed to be involved in the transcriptional control of further genes that encode, for example, a variety of neuropeptides including tachykinins. Regulation of IEG function (both by increased expression and phosphorylation) is brought about by a variety of second messenger pathways, and is a critical factor in controlling expression of many cellular changes. There are many IEG products which may be involved in nociception, and some of the long term molecular changes observed can be linked

with states of central sensitisation and persistent nociception (Coderre et al., 1993). The downstream targets of IEG mediated regulation are likely to include many genes with signalling or structural functions. One of these potential targets ubiquitin Carboxyl-terminal hydrolase-L1 UCH-L1) is discussed in Chapter 5.

1.20 Structural Reorganisation of the Dorsal Horn

In addition to alterations in biochemical processes there is evidence that the microanatomy of the dorsal horn can be modified following afferent nerve injury. Partial denervation, as a result of nerve injury, results in a substantial reduction in the synaptic contacts made by primary afferents with neurones in LII, and the subsequent rearrangement of the highly ordered laminar termination of primary afferents within somatotopically appropriate regions of the dorsal horn (Castro-Lopes et al., 1990; Woolf et al., 1992; Mannion et al., 1996). It has recently become apparent that regenerative changes occur following nerve injury. The discovery by Woolf et al. (1992) that large myelinated fibres sprout from LIII into LII in the dorsal horn following sciatic transection is of particular interest as this provides Aβ fibre input to a region of the dorsal horn normally involved in nociceptive processing by nociceptive C fibres. If the low-threshold mechanoreceptive afferent terminals that sprout into LII establish functional contacts with cells that normally would have a monosynaptic C-nociceptor input, this could lead to inappropriate responses to innocuous peripheral stimuli, perhaps an anatomical correlate of mechanical allodynia. This therefore suggests that the sprouting and reorganisation of primary afferent input could be related to the symptoms that sometimes follow nerve lesions (Woolf et al., 1992). Therefore, the already well demonstrated peripheral regenerative capacity of primary afferent neurones might be accompanied by an equally important central regenerative capacity, or plasticity, that in certain circumstances could be maladaptive (Woolf et al., 1992). Peripheral neuropathic pain may therefore be an expression in part, of an alteration in the circuitry of the spinal cord. However, it should be noted that the extent of sprouting that occurs following nerve injury is difficult to fully evaluate as there appears to be an increase in the uptake of the marker of myelinated fibres (Cholera toxin B-subunit (CTB)) following nerve injury (Tong et al., 1999).

1.21 Aims of the Study

This study had three main lines of investigation (a, b and c below) which correspond to chapters 3, 4 and 5 respectively.

(a) The role of the $VPAC_2$ receptor in the development and maintenance of the abnormal behavioural responses which occur following CCI in normal and mice lacking the $VPAC_2$ receptor ($VPAC_2R^{(-/-)}$).

(b) The role of cAMP-dependent protein kinase (PKA) in central neuropathic sensitisation in rats following CCI.

(c) The role of targeted protein degradation (via the ubiquitin-proteasome system) in central neuropathic sensitisation in rats following CCI.

CHAPTER 2: MATERIALS AND METHODS

2.1 Suppliers

Affiniti Research Products Ltd.

Mamhead Castle, Mamhead, Exeter, Devon, EX6 8HP, UK.

Amersham Life Sciences

Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK.

Astra-Zeneca

Alderley Park, Macclesfield, Cheshire, SK10 4TG, UK.

BDH Chemical Company

Merck House, Poole, Dorset, BH1 1TD, UK.

Beckton Dickenson UK Ltd (Transduction Labs)

Between Towns Road, Cowley, Oxford, OX4 3LY, UK.

Biolog. Lifesciences Institute

Forschung Labor und Biochemica-Vertrieb GmbH, PO Box 107125, D-28071, Bremen, Germany.

Biomol GmbH

Waldmannster, 35, D-22769, Hamburg, Germany.

Bio-Rad

Bio-Rad House, Marylands Avenue, Hemel Hempstead, UK.

Boehringer Mannheim Ltd.

Bell Lane, Lewes, East Sussex, BN7 11G, UK.

CellPath Ltd.

PO Box 101, Hemel Hempsted, Herts, HP3 8QE, UK.

Charles River

Manston Road, Margate, Kent, CT9 4LT, UK.

Chemicon International Ltd.

2 Admiral House, Cardinal Way, Harrow, HA3 5UT, UK.

Clark Electromedical Instruments Ltd. (Harvard Apparatus Ltd.)

Fircroft Way, Edenbridge, Kent, TN8 6HE, UK.

Fisher Scientific

Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RE, UK.

Genus Xpress (Ethicon)

10 Castings Court, Falkirk, KK2 9HJ, UK.

Gibco Life Technologies Ltd.

3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK.

Goodfellow Metals

Ermine Business Park, Huntingdon, PE29 6WR, UK.

HA West X-Ray Ltd.

41 Watson Crescent, Edinburgh, EH11 1ES, UK.

Hayman Ltd.

Eastways Park, Witham, Essex, CM8 3YE, UK.

Linton Instrumentation

No. 1 Forge Business Centre, Norfolk, IP22 1AD, UK.

Medical Instruments

Yale University, USA.

Merck Ltd.

Merck House, Poole, Dorset, BH15 1TD, UK.

NEN Dupont

Dupont NEN Research Products, S49 Albany Street, Boston, MA 02118, USA.

New England Biolabs

New England Biolabs, Knowl Piece, Wilbury Way, Hitchin, Herts, SG4 0TY, UK.

Oswel DNA Service

Institute of Biomolecular Sciences, University of Southampton, Southampton, UK.

Promega UK Ltd.

Delta House, Chilworth Research Centre, Southampton SO16 7NS, UK.

Rhône Merieux

Harlow, Essex, CM19 5TS, UK.

Roche Diagnostics

Bell Lane, Lewes, East Sussex, BN7 1L6, UK.

Santa Cruz Biotech Inc., (Autogen Bioclear, UK, Ltd.)

Holly Ditch Farm, Mile Elm, Calne, Wiltshire, SN11 0PY, UK.

Sigma-Aldrich Company Ltd

Fancy Road, Poole, Dorset, BH12 4QH, UK.

Stoelting

620 Wheat Lane, Wood Dale, IL60191, USA.

Thornton and Ross Ltd.

Linthwaite, Huddersfield, HD7 5QH, UK.

Unipath Ltd.

Priory Park, Bedford, MK44 3UP, UK.

2.2 Materials and Chemicals

2.2.1 Anaesthetics

Sodium pentobarbital (Sagatal, Rhône Merieux)

Halothane (Zeneca Ltd.)

α-chloralose (Sigma)

Urethane (Sigma)

2.2.2 Animals

Adult male Wistar rats (Charles River)

Adult male C57 / Bl6 wild-type and $VPAC_2R^{(-/-)}$ mice (kind gift donated by Professor Tony Harmar, Department of Neuroscience, University of Edinburgh).

2.2.3 Antibodies

The name and source of antibodies and their appropriate dilutions for Western blotting are shown in Table 2.1

Table 2.1 Table of Antibodies, Dilution and Source used in Western Blot Analysis

Antibody	Type	Dilution	Source
UCH-L1	Rabbit polyclonal	1:5000	Affiniti
GAPDH	Mouse monoclonal	1:750	Chemicon
PKA RI	Mouse monoclonal	1:250	Transduction Labs
PKA RIIα	Mouse monoclonal	1:100	Transduction Labs
PKA RIIβ	Mouse monoclonal	1:1000	Transduction Labs
PKA β-CAT	Rabbit polyclonal	1:500	Santa Cruz
Anti Mouse IgG	HRP-conjugated	1:2000	NEB
Anti Rabbit IgG	HRP-conjugated	1:2000	NEB

2.2.4 Drugs

The name and source of drugs used for ionophoresis and intrathecal administration are shown in Table 2.2

Table 2.2 Drugs used for Ionophoresis and Intrathecal Administration

Drug	Supplier	Application(s)	Action
MG-132	Affiniti	Ionophoresis i.t Injection Topical Application	Reversible Proteasome Inhibitor
Epoxomicin	Affiniti	i.t Injection Topical Application	Reversible Proteasome Inhibitor
Lactacystin	Affiniti	Ionophoresis i.t Injection	Irreversible Proteasome Inhibitor
Parthenolide	Biolog	i.t Injection	NF-κB Inhibitor
H-89	Biolog	i.t Injection	PKA Inhibitor
Myr-PKI (5-24)	R.Clegg*	i.t Injection	PKA Inhibitor
Control Myr-nonsense peptide	R.Clegg*	i.t Injection	No Known Activity
Rp-8-CPT-cAMPS	Biolog	i.t Injection	Competitive Inhibitor of PKA Activation by cAMP
Sp-5,6-DCl-cBIMPS	Biolog	i.t Injection	cAMP Analogue
Des(1-4)Arg^{16}-Ro 251553	P.Robberecht ♦	i.t Injection	$VPAC_2$ antagonist
Ro 251553	P.Robberecht ♦	i.t. Injection	$VPAC_2$ Agonist

* Kind gift donated by Roger Clegg, Hannah Research Institute, Ayr, Ayrshire, UK.

♦ Kind gift donated by Patrick Robberecht University of Brussels, Belgium.

2.2.5 Electrophysiology

Glass capillaries (Clark Electromedical Ltd)

Platinum wire (Goodfellow Metals)

Allyl isothiocyanate (mustard oil, Aldrich)

Liquid paraffin (Thornton and Ross)

Agar (Unipath)

2.2.6 Histological

OCT embedding matrix (CellPath)

Polysine®-coated microscope slides (BDH).

Mayer's haematoxylin (0.1% haematoxylin with stabilisers, Sigma)

Eosin (1% aqueous, Merck)

DEPX mounting medium (BDH)

Xylene (BDH)

Pontamine Sky Blue (BDH)

Bromophenol blue (BDH)

Coomassie blue R-250 (BDH)

Perfex mounting medium (CellPath)

2.2.7 Oligonucleotides

Oligonucleotide probes were synthesised and HPLC purified by Oswel Chemicals. Each probe was dissolved in approximately 1ml of sterile water, at a given concentration (μg/ml). The specificity of each probe was confirmed by data base screening of Genobase/EMBL sequences from which no significantly homologous alternative target sequences were recognised.

PKA RIα (48Mer) Accession number M17086

360 5' CGGTGGTGAAGGGCCGTCGGCGCCGCGGTGCTATCAGCGCTGAGG

3' CAGCCGCGGCGCCACGATAGTCGCGACTCC

TTTACACTGAGGAAGATGCTGCGTCCTACGTTAGAAAGG 3'444

AAATGTGACTCCTTCTAC 5'

PKA RIβ (48Mer) Accession number 321332

20 5' ATGGCCTCCCCATCATGCTTCCACTCAGAGGACGAGGACTCTCTGA

3' TCCTGAGAGACT

AAGGATGCGAGATGTACGTGCAGAAACATGGTATCCAGCAGGTGCTCAA

TTCCTACGCTCTACATGCACGTCTTTGTACCATAGG 5'

AGAATGC 3' 122

PKA RIIα (48Mer) Accession number J02934

1110 5' TAGATGTGATGAATCTCGGAGCCTTCTCAGTGTGATACCTAATCC

3' CGGAAGAGTCACACTATGGTTTAGG

TTCCAGTCAGCCACAAGAACACACCCAGAAAACAGACACGACAG 3' 1199

AAGGTCAGTCGGTGTTCTTGTG 5'

PKA RIIβ (50Mer) Accession numbers M12492; M21194

1250 5'AGCAAAAGTGTGGGGAAGAAAGCGCGTTAGTGAAGCAGTTACA

3' CGCGCAATCACTTCGTCAATGT

TAGCAGTGGTTAGTCCACCGAGGATGTGTTCGTGTAGATCAAGCA 3'1338

ATCGTCACCAATCAGGTGGCTCCTACAC 5'

PKA Cα (48Mer) Accession number X57986

560 5' ACCTTCTCATCGACCAGCAGGGCTATATTCAGGTGACAGACTTC

3'CCCGATATAAGTCCACTGTCTGAAG

GGTTTTGCCAAGCGTGTGAAAGG 3' 627

CCAAAACGGTTCGCACACTTTCC 5'

PKA Cβ (48Mer) Accession numbers D10770 D01144

40 5' CGGACTCCCGGGTCATGGGGAACACGGCGATCGCCAAGAAAGGC

3'TTGTGCCGCTAGCGGTTCTTTCCG

AGCGAAGTGGAGAGCGTGAAAGAA 3' 108

TCGCTTCACCTCTCGCACTTTCTT 5'

UCH-L1 (46Mer) Accession number NM_017237

290 5'

CGGGAACTCCTGTGGTACCATTGGGCTGATGCACGCAGTGGCCAAT

3'GGACACCATGGTAACCCGACTACGTGCGTCACCGGTTA

AACCAAGACAACCT 3' 340

TTGGTTCT5'

2.2.8 Photographic

Hyperfilm-β-max (Amersham)

LM1 Hypercoat nuclear emulsion (Amersham).

Kodak D-19 developer powder (HA West)

Ilford Hypam K5 Rapid fixer (Ilford)

Enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham)

Hyperfilm (ECL) autoradiography film (Amersham)

2.2.9 Sterile Surgery

Chromic catgut ligatures (4/0 Ethicon)

Coated vicryl sutures (4/0 Ethicon)

25G needle microsyringe (Beckton Dickenson)

Hibitane (chlorhexidine acetate, Zeneca)

Isopentane (BDH, Poole, UK)

Sterile gowns, biogel gloves, hats, face masks and drapes (Hospital Management and Supplies)

2.2.10 Western Blot Apparatus

10% Precast Tris-HCl ready gels (10% resolving gel, 4% stacking gel)(Bio-Rad)

Immuno-blot PVDF membrane (Bio-Rad)

Methanol (Hayman)

Blot absorbent filter paper (thick) (Bio-Rad)

Glacial acetic acid (Sigma)

Polyoxyethylenesorbitan monolaurate (Tween-20, Sigma)

Marvel dried skimmed milk

Lauryl sulphate (SDS, Sigma)

2.2.11 In situ Hybridisation Histochemistry Materials

Oligodeoxyribonucleotide probes (Oswel)

Deoxyadenosine [α-^{35}S]-triphosphate (specific activity <1250 Ci/mol, NEN Dupont)

Recombinant terminal deoxynucleotidyl transferase (Tdt) enzyme and tailing buffer (Promega)

Nu-Clean D25 disposable spin column (Scientific Imaging Systems)

Optiphase "Supermix" liquid scintillation cocktail (Fischer)

Absolute alcohol ANALAR grade (Hayman)

Acetic anhydride (0.25%) (BDH)

RNAsin ribonuclease inhibitor (Promega)

RNase ZAP (Sigma)

RNAse A (1 mg/ml) (Sigma)

Diethyl pyrocarbonate (DEPC) (Sigma)

Paraformaldehyde (Sigma)

Ethanolamine (10%, BDH)

5x Denhardt's solution (Sigma)

10% dextran sulphate (Sigma)

NaCl (Sigma)

Tris-HCl (10 mM, Sigma)

EDTA (1 mM, Sigma)

Salmon sperm DNA (Sigma)

Yeast tRNA (Sigma)

Glycogen (Gibco)

Ammonium acetate (Sigma)

Triethanolamine (TEA, Merck)

Sodium phosphate $NaH_2(PO_4)_3$ (Monobasic, Sigma)

Sodium phosphate $Na_2H(PO_4)_3$ (Dibasic, Sigma)

Formamide (Sigma)

Mixed bed resin (Sigma)

20xSSC (Gibco)

Dithiothreitol (DTT, Sigma)

2.2.12 Miscellaneous

ART pipette tips (RNase free, Promega)

2ml eppendorf tubes (Merck)

1M sodium chloride solution (Sigma)

0.5% aqueous dimethylformamide (Biomol).

2.2.13 Stock Solutions for In Situ Hybridisation and Western Blotting

RNase-free ddH_2O

DEPC 1% (v/v) in ddH_2O, autoclave

Phosphate-buffered saline (PBS)

50mM $NaHCO_3$, pH 7.5; 150mM NaCL

4% paraformaldehyde (PFA) in PBS

0.1M PBS, 4% (w/v) (fresh) paraformaldehyde

Triethanolamine (TEA)

10M stock TEA diluted to 0.1M with DEPC H_2O pH8

2X Hybridisation buffer

Dextran sulphate 10% (w/v), NaCl (600mM), Tris pH 7.6 (10mM), EDTA (1mM), Denhardt's solution (1% solution of bovine serum albumin (BSA), Ficoll and polyvinyl pyrrolidone (PVP)) 0.1% (w/v), salmon sperm DNA 0.01% (w/v), Bakers yeast tRNA 0.005% (w/v), glycogen 0.0005% (w/v) and RNAsin ribonuclease inhibitor (Promega)

De-ionised formamide

Formamide was de-ionised using mixed bed ion exchange resin

SDS lysis buffer (5X Stock)

0.625M Tris-Cl, pH 6.8; 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 50% (v/v) glycerol

Electrophoresis buffer

1M Tris base, 0.1M glycine (+SDS)

Coomassie staining reagent

0.1% (w/v) Coomassie brilliant blue R-250, 25% (v/v) isopropanol, 10% (v/v) acetic acid

Destaining reagent

10% (v/v) acetic acid, 5% (v/v) isopropanol

Protein transfer buffer

0.1M Tris base, 0.5M glycine, 0.01% (w/v) SDS

Blocking buffer

1X PBS, 4% (w/v) non-fat milk (Marvel)

Wash buffer

1X PBS, 0.1% (v/v) Tween 20

2.3 Methods

2.3.1 Chronic Constriction Injury (CCI) to the Sciatic Nerve

All experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986. Adult male Wistar rats (200-350g) were anaesthetised with sodium pentobarbital (Sagatal 0.06ml / 100g, i.p.) and supplemented with halothane / O_2 (2-3%). Under aseptic conditions, the right sciatic nerve was exposed proximal to the trifurcation, at a mid-thigh level, and 4 (4 / 0, Ethicon) chromic cat gut ligatures were tied to loosely constrict the nerve (chronic constriction injury (CCI)), as viewed under x40 magnification (Bennett and Xie, 1988). The overlying muscle and skin were closed with sutures (4 / 0, Ethicon) and the animals were allowed to recover for 72 hours before reflex testing recommenced. CCI surgery was also carried out in mice for some experiments (Chapter 3). Adult male C57 / Bl6 mice were anaesthetised with Sagatal (0.01ml / 100g, i.p.) and supplemented with halothane / O_2 (0.5-1.5%). Under aseptic conditions, the right sciatic nerve was exposed proximal to trifurcation, at the mid-thigh level, and 3 (5 / 0, Ethicon) chromic cat gut ligatures were tied to loosly constrict the nerve (CCI), as viewed under x40 magnification. The overlying muscle and skin were closed with sutures (5 / 0, Ethicon) and the animals allowed to recover for 72 hours before reflex testing recommenced. Sham-operated rats and mice underwent the same surgical procedure, but no ligatures were placed around the nerve.

2.3.2 Behavioural Tests

Behavioural signs representing three different components of neuropathic pain were investigated: thermal hyperalgesia, mechanical allodynia and cold allodynia. Behavioural testing was carried out prior to surgery to establish a baseline for comparison to post surgical values. Only nerve-injured animals that showed clear signs of thermal hyperalgesia, cold and mechanical allodynia were used for further study. In practise only 1 in 25-30 animals failed to develop sensitisation and whenever these cases were further investigated, technical problems with the nerve ligation were identified.

2.3.2.1 General Observations

Inspections were regularly made for signs of autotomy, which was rarely observed. General observation of the animals posture and condition of the affected limb were also made.

2.3.2.2 Thermal Hyperalgesia

Thermal hyperalgesia was monitored using noxious radiant heat (30-55°C) applied to the mid-plantar glabrous surface of the hind paw using Hargreaves' thermal device, (Linton). The withdrawal response latency was characterised as a brief paw flick recorded to the nearest 0.1s, and a standard cut-off latency of 20s was employed to prevent tissue damage. Testing was repeated a minimum of 5 times with not less than 5 min between trials to avoid any sensitisation of the paw.

2.3.2.3 Mechanical Allodynia

Mechanical allodynia was measured as the threshold for paw withdrawal in response to normally innocuous graded mechanical stimuli applied to the mid-plantar glabrous surface of the hind paw using Semmes-Weinstein calibrated von Frey filaments (Stoelting). Each filament was applied perpendicularly to the mid-plantar surface of the foot until it started to bend. This was repeated 10 times at a frequency of approximately 1Hz. The filaments were applied in ascending order and a response characterised as a robust paw flick. Threshold was defined as the force that caused foot withdrawal 5 times in every 10 applications.

2.3.2.4 Cold Allodynia

To detect the presence of cold allodynia, rats were placed in a perspex box with an elevated aluminium floor covered with iced water, sufficient to immerse both glabrous and hairy skin of the hind paw (3-4°C; Bennett and Xie, 1988). Rats were allowed 10s to acclimatise once placed in the box. The number of seconds the animal raised its hind paw above the water over a 20s period was recorded. This was repeated 4 times at 10 min intervals to establish a mean suspended paw elevation

time (SPET) for each rat. Animals determined as being at the peak of neuropathy, 12-15 days following CCI surgery, were then used in further study.

2.3.3 Nerve Preparation for Analysis

2.3.3.1 Dissection and Sampling

To excise the nerve, mice were deeply anaesthetised with sodium pentobarbital and perfused transcardially with 0.1M PBS (containing 3mM sodium nitrite and 1000u heparin, pH 7.4) before being perfused with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1% sodium cacodylate buffer (pH 7.3). The sciatic nerve was then removed and fixed for 2h in the same fixative and post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer and embedded in araldite.

2.3.3.2 Myelinated Fibres

1μm transverse sections of sciatic nerve (tibial branch) stained with toluidine blue were examined using 'Image 1.44' software (NIH). The cross sectional area of the tibial nerve was measured at x100 magnification and 5 randomly generated regions on each nerve section were chosen using a 4x4 grid. For analysis, 30 myelinated fibres closest to the centre of the screen were selected and the axon areas, external areas, and G ratios (axon diameter / external diameter) were measured to quantify myelin thickness.

2.3.3.3 Unmyelinated Fibres

80nm transverse sections of the sciatic nerve were mounted onto copper slot grids and examined in a transmission electron microscope. 25 random areas were photographed at 2300x magnification and then printed at a final magnification of x5750, which was used to count all the myelinated and unmyelinated fibres. For analysis, 5 negatives were chosen at random and scanned using a flat bed scanner for computer analysis using 'Image 1.44'. The axon diameters / areas of 30 unmyelinated fibres were measured from each of the micrographs, the fibres being

selected from clusters consisting solely of unmyelinated fibres. The frequency of myelinated fibres was scored at 1 μm intervals from 1-10 μm and that of unmyelinated fibres at 0.1 μm intervals from 0.1 – 2.7 μm.

2.3.4 Intrathecal Drug Administration

Prior to intrathecal injection, baseline measurements were made in rats that had undergone CCI surgery as previously described (section 2.3.1). Rats at the peak of neuropathy showing behavioural alterations indicative of neuropathic pain were selected for i.t. administration experiments. Following induction of brief anaesthesia (halothane / O_2 1%) the space between the lumbar vertebrae (L5-6) was identified and a 25G needle microsyringe orientated between the vertebrae and into the intrathecal space. A 50 μl (rats) or a 30 μl (mice) bolus was injected and the needle slowly removed. Testing began 15 minutes post-injection, and was carried out as described previously (sections 2.3.2.2, 2.3.2.3 and 2.3.2.4). Testing continued for a minimum of 90 minutes and until recovery to pre-injection values.

2.3.4.1 Control Experiments

50 μl injections of Pontamine Sky Blue were carried out and a laminectomy was carried out post-mortem to identify the location of the dye. Drug injections were performed blind to eliminate bias and saline injections were carried out in parallel with drug injections as a further control (data not shown).

2.3.5 Surgical Procedure for Electrophysiological Preparation

Details of all reagents can be found in section 2.2. Rats used for electrophysiological recordings underwent CCI surgery as outlined in section 2.3.1. Only nerve injured animals which showed strong signs of thermal hyperalgesia, mechanical and cold allodynia (sections 2.3.2.2, 2.3.2.3 and 2.3.2.4) were used for electrophysiological recordings. Normal unoperated rats were also used routinely for control purposes. Rats were anaesthetised with intravenous α-chloralose (60mg / kg) and urethane (1.2mg / kg) following induction with halothane to allow jugular vein cannulations.

Supplementary doses of α-chloralose were given as required. A tracheotomy was performed to maintain an unobstructed airway, and the rat was allowed to breath freely, although oxygen (0.1 1 / min) was passed over the end of the cannula to enrich the inspired air. Core temperature measured by a rectal probe was maintained at 37-38°C by means of a thermostatically controlled heated blanket. The animal was mounted in a rigid stereotaxic frame and spinal segment L2 was identified by locating the floating rib and the thoraco-lumbar spinal column was supported using 3 pairs of swan-necked clamps on alternate segments, with the middle clamp supporting L2. A pool was made with skin flaps around the area of interest and a dorsal laminectomy performed under x 12.5 magnification to expose segments L1-L4. To improve the stability of the preparation for extracellular recording, agar solution (2% in 0.9% saline solution) at 39°C was injected under the most rostral vertebrae and then poured over the entire pool, including the spinal cord. Once cooled, a core of agar was removed to expose the area of cord from which the recordings would be made, the dura was carefully cut and a pool of 37°C liquid paraffin applied to the exposed area to prevent dehydration.

2.3.6 Extracellular Recording

Extracellular recordings were made from single dorsal horn neurones using the central barrel (4M NaCl, pH 4.0-4.5) of a 7 barrelled glass microelectrode, with tip sizes 4.0-5.5μm. One side barrel contained 1M NaCl (pH 4.5) for automatic current balancing using a Neurophore BH2 ionophoresis system (Medical Systems Corporation). A second side barrel contained 2% Pontamine Sky Blue in 0.5M sodium acetate for histological marking of recording sites (section 2.3.6.1). The remaining barrels contained the drugs for ionophoresis. Recordings were made at depths of 0-1000μm from the spinal cord surface (as monitored by the microdrive), and cellular activity was observed on the oscilloscope screen (Tektronix). The output activity from the oscilloscope was continually monitored using a D130 digitimer spike processor, with the spike discriminator adjusted so that the activity of one neurone was isolated (as viewed by markers on the oscilloscope) for counting. Neuronal firing rate was continuously plotted on-line via a customised analysis

program (Scap 90; Dr M Dutia, Department of Physiology, University of Edinburgh) on a Dell PIII 500 workstation.

2.3.6.1 Identification of Neuronal Receptive Fields

Neuronal receptive fields were identified by manually brushing the ipsilateral hind limb, whilst lowering the electrode into the spinal cord using a microdrive. Neurones were further examined using noxious pinch and heat (48°C for 10s). The heat stimulus was applied using a ramp driven Peltier device (Medical Instruments) with a contact area of $1cm^2$. The Peltier probe, while in contact with the animals skin, was raised from a resting temperature of 32°C to 48°C (ramp rate 5°C / s) to be held at 48°C for 10s. The neurones were then classified according to their sensory input and those neurones classified as being multireceptive (i.e those that responded to noxious heat, pinch and innocuous brush) were used for this study. Extracellular recordings were made from single, multireceptive neurones (LIII-V), ipsilateral to the nerve injury, in neuropathic animals and bilaterally in unoperated control rats.

2.3.6.2 Quantification of Neuronal Responses to Cutaneous Stimuli

The effects of inhibitors were examined on evoked responses of neurones to sensory stimulation in both normal and neuropathic animals. Sustained activation of neurones was obtained by the following stimuli: (i) rotating motorised innocuous brush for low threshold mechanical stimulation, (ii) intense cold stimulus provided by a Peltier device (5°C for 10s, from 32°C (ramp rate 5°C / s), over a surface area of 1 cm^2 repeated every 1-2 min to give reproducible peaks of activity, (iii) topical application of the C-fibre selective chemical algogen, mustard oil (allyl isothiocyanate, Aldrich), 7.5% solution in paraffin oil, applied up to 7 times at 5 min intervals), until a steady state elevated firing (3-45Hz) was maintained. The specific proteasome inhibitors lactacystin and MG-132 (both in 0.5% dimethylformamide at concentrations of 500 μM and 200 μM respectively) were ejected using positive currents of 5-60 nA and a retention current of -12 nA was applied to each side barrel when not in use, to minimise drug leakage. The resistance of the side barrels was

monitored regularly and electrodes with resistance values in excess of 45 MΩ were rejected. Drugs were ejected ionophoretically in a step-wise manner (usually by 10 nA, over a range of 5-60nA), every 1 to 2 min, until clear effects on the neuronal firing rate were observed, or if none was observed by 60 nA after 2 min duration, the drug application was terminated. For the majority of neurones, drugs were tested on responses to all three sensory stimuli.

2.3.6.3 Analysis of Electrophysiological Results

Action potential discrimination enabled data to be digitised and downloaded onto a computer to give a record of cell firing rate against time (spikes / s). After subtraction of the 'background' activity, the firing rate following drug application was expressed as the percentage change from control responses of the neurone prior to drug application. The change in activity was expressed as a percentage of the control value for each cell, and then pooled to find the mean ± SEM.

2.3.7 In situ Hybridisation Histochemistry

2.3.7.1 Preparation of RNase-free ddH_2O, Buffers and Apparatus

Buffers used for RNA analysis are generally treated with diethyl pyrocarbonate (DEPC) to inactivate ribonuclease (RNase) enzymes before being autoclaved. RNase-free ddH_2O was prepared by adding DEPC to 1% (v / v) in ddH_2O, mixing vigorously and incubating overnight at room temperature before autoclaving. Pipette tips and 2ml eppendorf tubes were autoclaved to eliminate RNase contamination. All apparatus used during in situ hybridisation experiments were treated with RNase ZAP and rinsed with DEPC-treated ddH_2O prior to use.

2.3.7.2 Spinal Cord Dissection Prior to Insitu Hybridisation Histochemistry and Western Blot Analysis.

Neuropathic, sham operated and age-matched control rats were deeply anaesthetised with halothane / O_2 (4% for induction and maintenance). Under aseptic conditions a laminectomy (L3-L6) was performed for spinal cord removal as described previously

(section 2.4.4). Tissue was removed intact for in situ mounted on a cryostat chuck with OCT embedding matrix, and snap frozen in isopentane at -40 to -45°C. For Western blot analysis spinal cord samples were hemisected down the midline. To split the spinal cord medially a scalpel blade was used to slice from L3-L5 and each hemisected section removed separately and placed immediately in a sterile eppendorf on ice.

2.3.7.3 3' Labelling of Oligonucleotides with Deoxyadenosine [^{35}S]-Triphosphate

The oligonucleotides were 3' end-labelled with deoxyadenosine [^{35}S]-triphosphate (specific activity <1250 Ci / mol; NEN Dupont) using terminal deoxynucleotidyl transferase (Tdt) (Promega), and potassium cacodylate tailing buffer (Tdt buffer) (Promega) and DEPC H_2O (1hr, 37°C). The labelled oligonucleotide was then separated from unincorporated oligonucleotide using a Nu-Clean D25 disposable spin column (Scientific Imaging Systems). Samples were taken before and after this purification stage for scintillation counting. This allowed the percentage incorporation of oligonucleotide / radiolabel to be calculated (oligonucleotides were selected that showed 40-60% incorporation).

2.3.7.4 Tissue Preparation Prior to In situ Hybridisation Histochemistry

For details on tissue dissection refer to section 2.3.7.2. Frozen transverse sections of spinal cord (10 μm) were taken at -19°C in a cryostat and thaw-mounted onto Polysine®-coated microscope slides. Tissue sections were equilibrated to room temperature and then post-fixed for 10 min in 4% PFA / 0.1 M phosphate-buffered saline (pH 7.4). They were then pre-hybridised in ethanolamine (10%) (0.1M TEA and 0.025M acetic anhydride for 10 min at room temperature), before being dehydrated in ascending concentrations of ethanol (50, 60, 70, 90, 95 and 100%), each buffered with ammonium acetate, and air dried.

2.3.7.5 Hybridisation

Previously prepared stock solutions of hybridisation buffer and deionised formamide (section 2.2.13) were combined (1x hybridisation buffer / 40% deionised formamide). The radiolabelled oligonucleotides were added in a relative volume to give a final count number of $2x10^6$ cpm / 100µl hybridisation buffer (optimised to establish clearest signal:background ratio). The resulting hybridisation mix was heated to 60-70°C (10 min) to eradicate dimer formation, cooled on ice (1 min) before adding the reducing agent DTT (10mM). Sections were then covered with this solution and placed in sealed containers at 50°C (15-20 h) which were kept humid with equal volumes of 4x SSC and deionised formamide.

2.3.7.6 Post-Hybridisation Washes

After hybridisation the sections were washed in 2 x SSC, 1 x SSC and 0.5 x SSC (all 2 h each at 40°C), to remove any excess hybridisation buffer or non-specifically bound oligonucleotides. The slides were then dehydrated in ascending ethanol concentrations, after which they were air dried.

2.3.7.7 Photographic Development of Sections

The sections were then apposed to Hyperfilm-β-Max to provide an estimate for subsequent exposure time after dipping in photographic emulsion. After 3 - 7 weeks they were developed at 15°C using Kodak D19 developer (4 min) and Ilford Hypam rapid fixer (5 min), then counterstained in 0.1% Mayer's haematoxylin, and 0.1% eosin, mounted using DEPX mounting medium, and coverslipped.

2.3.7.8 Stringency Controls for In Situ Hybridisation Histochemistry

Controls used to demonstrate specificity of the respective oligonucleotide consisted of (i) pretreating sections with RNAse A (1 mg / ml) for 1 h prior to hybridisation, (ii) co-incubation of the ^{35}S-labelled oligonucleotide in the hybridisation medium with a 100-fold excess of unlabelled oligonucleotide.

2.3.7.9 Image Analysis

Cell Counts

The number of cells positively hybridised within lateral and medio-lateral locations of laminae I - V was calculated at x40 magnification (total grid area 175 x 175 μm). Cells were considered to be positively-labelled if the silver grains showed a dense pattern around the nucleus and were 5-fold denser than a typical non-expressing cell within the same field area or background levels. The total number of positively hybridised cells was determined for each spinal cord section, and these values used to calculate the mean number of positively hybridised cells per unit area, in the lumbar spinal segments L3-6.

Silver Grain Counting

To assess any changes in the relative expression of mRNA following chronic constriction injury, the mean silver grain density per positively labelled cell was measured using 'Image 1.44' software (Improvision) with video input from a CCD camera (Sony, Japan) mounted on a Zeiss Axioscope microscope (x40 magnification). For each section, counts were made both ipsilateral and contralateral to the nerve injury in lateral and medial zones. A minimum of 5 positively hybridised cells were counted in each region. A pixel count was obtained, which was converted to silver grain number via a pre-determined calibration procedure. The mean number of silver grains per cell was calculated for each section, to allow calculation of the mean number of silver grains per cell. In total, 500 positively hybridised cells were counted within one side of the spinal dorsal horn for each animal.

2.3.8 Tissue Preparation Prior to Western Blotting

For details on tissue dissection refer to section 2.2.13 and section 2.2 for solutions and buffers. Immediately following tissue extraction, samples were individually weighed and transferred to a 1ml homogeniser containing ten volumes of ice cold 2x sample buffer. Once homogenised, samples were heated to 100°C for 5 min. Genomic DNA / RNA was sheared by passing the lysate several times through a

narrow gauge hypodermic needle and syringe prior to centrifugation at 13,000 x g for 10 min at room temperature. The supernatant was transferred to a fresh microcentrifuge tube and either analysed by gel electrophoresis, or stored at -40°C.

2.3.8.1 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis was performed using the Tris-glycine discontinuous buffer system described by Laemmli *et al*, (1970), for protein separation. 10-15 well 10% pre-cast gels were used for the experiments shown. The gel tank was filled with electrophoresis running buffer (1x). Samples and molecular weight marker proteins were loaded into the wells of the stacking gel and electrophoresed at a constant voltage of 100V, until the samples passed into the resolving gel. At this point the voltage was raised to 150V, and run for approximately 70 minutes until the dye front had run off the end of the resolving gel. Following electrophoresis the protein was either visualised by Coomassie staining (section 2.3.8.2) or transferred to PVDF membrane (section 2.3.8.3).

2.3.8.2 Coomassie Staining

Polyacrylamide gel slabs were transferred into an excess volume of Coomassie staining solution and agitated on an orbital mixer for 1h at room temperature. Protein bands were visualised following several changes of excess Coomassie destaining solution.

2.3.8.3 Electrotransfer of Protein from SDS-PAGE Gels to PVDF

PVDF membranes were soaked in methanol for 1 minute. Following SDS-PAGE, the gel, PVDF, pre-cut filter paper, and fibre pads were soaked in SDS buffer for 2 minutes. The Bio-Rad Protein II gel transfer apparatus was assembled, ensuring no air bubbles were present.

2.3.8.4 Electro-Transfer to PVDF

The gel, membrane and filter paper sandwich was then placed in the electrophoresis buffer chamber, along with an ice pack, and filled with transfer buffer. A constant voltage of 100V was applied for 45 min.

2.3.8.5 Western Blotting

Following electrophoretic transfer, the PVDF membrane was rinsed briefly in PBS tween (PBS-T). Blocking was performed in 4% Marvel dried skimmed milk in PBS-T for either 60 minutes at room temperature or overnight at 4°C on a rocking platform. Following blocking the membrane was briefly rinsed with PBS-T and incubated with the primary antibody at the required dilution (Table 2.1), in PBS-T with 4% Marvel at room temperature for 90 minutes. After six X 5 minute washes in PBS-T at room temperature, the PVDF was incubated with the Horseradish peroxidase (HRP)-conjugated secondary antibody (Table 2.1) in PBS-T with 4% Marvel for 40 minutes. Finally the PVDF was washed as above with the addition of a final rinse in PBS only.

2.3.8.6 Enhanced Chemiluminescence Detection

To detect bound antibody, membranes were incubated with a minimal volume of enhanced chemiluminescence (ECL) detection reagent for 1 minute at room temperature as described in the manufacturers protocol. PVDF was placed between two transparency sheets before exposure to Hyperfilm ECL autoradiography film. Several exposures were carried out to ensure linear exposure for direct comparison between bands.

2.3.9 Ex vivo PKA Activity Assays

Following lumbar L3-6 laminectomy under anaesthesia, rats, (normal; n=3, neuropathic; n=3,) were treated by topical application of agents to the dorsal surface of the spinal cord. Epoxomicin (15 μM), MG-132 (100 μM) or vehicle (0.5% dimethylformamide in saline) was applied in a volume of 500μl for 1 hour before

rapid removal to cold buffer on ice. cAMP-evoked PKA enzymic activity and constitutive activity, (thought to reflect PKA previously activated in situ) were measured by the following procedure; a modification of those of Roskoski (1983) and Corbin (1983). Hemisected spinal cord samples were rapidly homogenised on ice in 20 mM Na HEPES pH 7.5 with 5% glycerol, 0.25% BSA, 1mM EGTA, 1mM dithiothreitol, 1mM AEBSF (4-(2-aminoethyl) benzene sulphonyl fluoride), (Alexis Corporation), 2 $\mu g.ml^{-1}$ aprotinin, 10 $\mu g.ml^{-1}$ leupeptin, 2 $\mu g.ml^{-1}$ pepstatin, 50 $\mu g.ml^{-1}$ soybean trypsin inhibitor, 25 mM Na α-glycerophosphate, 1mM Na orthovanadate, 1mM NaF, 1 μM calyculin A, 1 μM cypermethrin (Calbiochem, Nottingham, UK) and 500 μM isobutyl methylxanthine. All reagents were from Sigma unless otherwise indicated. Aliquots of homogenate were incubated for 10 min at 30°C (linear range of assay) with 100 μM kemptide as substrate, 100 μM ATP (with [^{33}P] α-ATP to 0.2 μCi / tube; NEN-DuPont, Dreiech, Germany), 10 mM $MgCl_2$ and 10 μM cAMP and / or 1 μM PKI $_{6-22}$ amide (Calbiochem) as appropriate. Assays were terminated with cold TCA (to 10%), centrifugation and spotting of the supernatant onto P81 phospho-cellulose paper. Samples were washed extensively in 75 mM H_3PO_4 and dried before scintillation counting. Positive controls were carried out with purified bovine heart PKA catalytic subunit and each homogenate was assayed in parallel for cAMP-evoked activity (in the absence or presence of PKI $_{6-22}$ amide), constitutive activity (in the absence or presence of PKI $_{6-22}$ amide) and zero time incubation blanks. Authentic (PKI $_{6-22}$ amide-sensitive) PKA-activity was always 86-97% of the recorded activity for constitutive and cAMP-evoked conditions and zero time blanks were always <2% of total activity.

CHAPTER 3: THE ROLE OF THE $VPAC_2$ RECEPTOR IN NEUROPATHIC PAIN

3.1 VIP and PACAP

The 28 amino-acid peptide Vasoactive Intestinal Polypeptide (VIP) was isolated from porcine small intestine (Said and Mutt, 1970; Mutt and Said, 1974). Radioimmunochemical studies have revealed a widespread distribution of VIP throughout the peripheral and central nervous system (Gibson et al., 1981; Larsson et al., 1976; Yaksh et al., 1988), suggesting that it may also play an important role as a neurotransmitter.

Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) was isolated from ovine hypothalamus (Miyata et al., 1989). There are two variants, PACAP-38 (a 38 amino-acid polypeptide) and the C-terminally truncated PACAP-27 (Miyata et al., 1990). PACAP is a member of the VIP/secretin/glucagon family of structurally related peptides, and PACAP shows 68% homology with VIP at its N-terminal (Arimura, 1992). PACAP is widely distributed throughout the PNS and CNS where it may act as a neurotransmitter/neuromodulator (Arimura, 1992; Arimura and Shioda, 1995; Ghatei et al., 1993; Moller et al., 1993). The distinct anatomical distribution of VIP and PACAP within the CNS has lead to the suggestion that these peptides may serve as sensory transmitters and so may play an important role in the modulation of somatosensory processing. (Arimura and Shioda, 1995; Ghatei et al., 1993; Gibson et al., 1981; Lamotte and de Lanerolle, 1986; Larsson et al., 1976; Yaksh et al., 1988).

The close sequence homology between VIP and PACAP means that they share the same receptor binding sites (Harmar and Lutz, 1994). VIP and the 2 forms of PACAP are recognised by a family of three receptors, namely the $VPAC_1$ (Ishihara et al., 1992), $VPAC_2$ (Lutz et al., 1993) and PACAP (Hosoya et al., 1993) receptors.

3.2 $VPAC_1$ and $VPAC_2$ Receptors

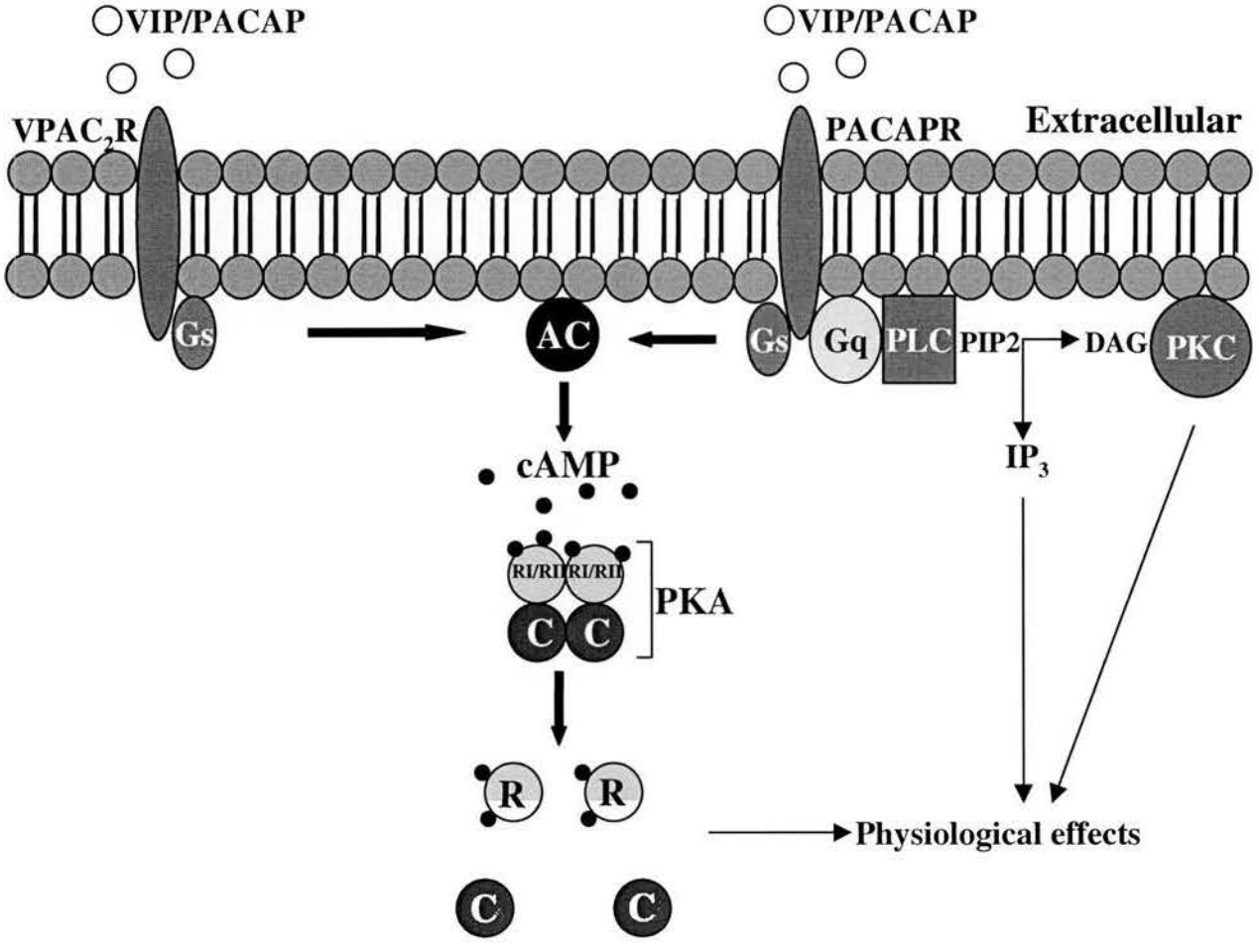
$VPAC_1$ and $VPAC_2$ receptors are seven trans-membrane domain G-protein coupled receptors. They are positively coupled to the α-subunit of the adenylate cyclase stimulating G protein (Gs), and so upon activation stimulate the activity of adenylate cyclase (Figure 3.1) (Ishihara et al., 1992; Lutz et al., 1993). Both receptors bind VIP and PACAP with similar high affinity, although they are differentially distributed (Cauvin et al., 1991; Usdin et al., 1994). The $VPAC_1$ receptor is located predominantly in liver, small intestine and certain areas of the brain (cortex, hippocampus and olfactory bulb). The $VPAC_2$ receptor however, is primarily located in the thalamus, hippocampus suprachiasmatic nucleus (SCN) and hypothalamus, but is also present in superficial dorsal horn (Sheward et al., 1995; Lutz et al., 1993).

3.3 PACAP Receptor

The PACAP receptor displays much greater affinity for the 2 forms of PACAP than VIP (Hashimoto et al., 1993; Shivers et al., 1991), and has a widespread distribution including the CNS (Cauvin et al., 1991; Masuo et al., 1991). The PACAP receptor is coupled to dual signalling cascades involving the Gs and Gq proteins (Figure 3.1) and so upon activation can stimulate both adenylate cyclase and phospholipase C (PLC) signal transduction cascades.

Figure 3.1 VIP/PACAP Receptor Activation of Second Messenger Pathways

Schematic representation of the VIP/PACAP receptors and the signal transduction pathways associated with their activation. The $VPAC_1$ and $VPAC_2$ receptors are both positively-coupled to the α-subunit of the adenylate cyclase stimulating G protein (Gs) which activates adenylate cyclase (AC) following the agonist-induced exchange of guanosine tri-phosphate (GTP) into guanosine diphosphate (GDP). Adenylate cyclase subsequently converts adenosine triphosphate (ATP) into cAMP, which in turn activates PKA, to result in the phosphorylation of a number of proteins and receptors within the cell. Both VIP and PACAP can activate the $VPAC_1$ and $VPAC_2$ receptors with similar binding affinity. The PACAP receptor is coupled to dual signalling cascades involving the Gs protein which activates AC as above, and the Gq protein, which activates the phospholipase C (PLC) pathway converting phosphatidyl inositol 4,5-diphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3). This causes the intracellular release of Ca^{2+} ions from intracellular ca^{2+} stores, and diacyl glycerol (DAG) which induces phosphorylation of intracellular proteins through activation of PKC. Increases in the intracellular levels of ca^{2+} ions have been linked with a number of physiological effects, including the activation of immediate early genes and subsequently changes in receptor and gene expression within the cell.


VIP/PACAP
VIP/PACAP
VPAC2R
PACAPR
Extracellular
Gs
AC
Gs
Gq
PLC
PIP2
DAG
PKC
cAMP
IP3
RI/RII
RI/RII
PKA
C
C
R
R
Physiological effects
C
C

3.4 VIP/PACAP Mediated Spinal Somatosensory Transmission

The tachykinins SP and CGRP appear to lose their excitatory role following nerve transection and it is believed that VIP could adopt the excitatory role of SP following nerve injury (Wiesenfeld-Hallin et al., 1990). Intrathecal administration of VIP leads to a facilitation of nociceptive flexor reflexes, and when applied ionophoretically VIP causes a marked excitation of both the rat trigeminal nucleus caudalis neurones as well as neurones of the rat and cat spinal cord (Dickinson et al., 1999; Salt and Hill, 1981; Dickinson et al., 1997; Jeftinija et al., 1982). VIP appears not to play a role in C-fibre induced spinal sensitisation (wind up), in normal animals. However, intrathecal administration of the VIP antagonist (Ac-Tyr, D-Phe^2)-GRF(1-29)-NH_2 significantly inhibits C-fibre facilitation of the flexor reflex following CCI (Xu and Wiesenfeld-Hallin, 1991; Wiesenfeld-Hallin et al., 1990). Therefore it appears that VIP adopts a more important role in spinal somatosensory processing following nerve injury.

PACAP displays a generally excitatory effect and ionophoretically applied PACAP displays an excitatory effect on single dorsal horn neurones (Dickinson et al., 1999). However, the behavioural effects of PACAP are not as obvious as VIP. Some evidence demonstrates an antinociceptive effect at the spinal level as intrathecal administration of PACAP-27 suppresses wind up in normal rats (Zhang et al., 1993). Concomitant data has demonstrated that pre-treatment of rats with intrathecal PACAP decreased formalin induced flinching behaviour (Yamamoto and Tatsuno, 1995). In contrast, flexor reflex studies in decerebrate spinalised rats revealed that intrathecal PACAP-27 produced dose dependent facilitation of the flexor reflex (Xu and Wiesenfeld-Hallin, 1996). In agreement with these findings, another study has since shown that low doses of intrathecal PACAP-38 produces a dose dependent decrease in tail flick latency to noxious heat in mice whereas higher doses produced a dose dependent increase in biting and scratching behaviours indicative of pain behaviours (Narita et al., 1996).

3.5 Roles of $VPAC_1$, $VPAC_2$ and PAC_1 Receptors in Spinal Sensory Processing

$VPAC_1$, $VPAC_2$ and PAC_1 receptors appear to play an important regulatory role in the transmission of sensory information at the spinal cord level (Dickinson and Fleetwood-Walker, 1999). Ionophoretically applied inhibitors of both $VPAC_1$ and PAC_1 receptors exert a generalised modulation of dorsal horn neurones in normal, anaesthetised rats, inhibiting both innocuous brush evoked activity and sustained C-fibre evoked activity (Dickinson et al., 1999). In contrast, ionophoretic application of the $VPAC_2$ receptor inhibitor (Des-1-4-Arg^{16}-Ro25-1553) inhibited only the noxious C-fibre evoked activity induced by topical application of mustard oil (Dickinson et al., 1999).

Following CCI, all three receptor inhibitors have negligible effects on innocuous brush evoked activity of dorsal horn neurones (Dickinson et al., 1999). $VPAC_1$ and PAC_1 receptor antagonists have been shown to attenuate cold-induced neuronal firing, implicating them in the development and/or maintenance of cold allodynia following nerve injury (Dickinson and Fleetwood-Walker, 1999). Antagonists of all three receptor subtypes inhibited mustard oil-induced activity, particularly antagonists of the $VPAC_2$ receptor subtype (Dickinson et al., 1999). The altered involvement of these receptors following CCI is likely to result, in part, from their altered expression in the dorsal horn as revealed by in situ hybridisation histochemistry. In fact $VPAC_2$ receptor mRNA is increased in the dorsal horn following CCI (Dickinson et al., 1999). In addition, ionophoretically applied Ro25–1553, a $VPAC_2$ receptor agonist greatly increases neuronal excitation following CCI (Dickinson et al., 1999) thus highlighting the potential importance of the $VPAC_2$ receptor in the development and/or maintenance of neuropathic pain following CCI.

3.6 Aims of The Experiments

From the most recent functional studies carried out, using novel selective antagonists, it appears that the $VPAC_2$ receptor antagonists could serve as selective analgesics, strongly reducing polymodal C-fibre mediated pain in normal and

neuropathic pain states. These experiments sought to further characterise the possible contribution made by the $VPAC_2$ receptor to the development and maintenance of the abnormal pain behaviours indicative of neuropathic pain following CCI in normal and $VPAC_2R^{(-/-)}$ mice, and also sham-operated mice.

Sciatic nerve morphology was assessed for control purposes, to ensure that any behavioural differences between wild-type and $VPAC_2R^{(-/-)}$ mice were not caused by developmental abnormalities in peripheral nerve morphology. In unoperated wild-type and $VPAC_2R^{(-/-)}$ mice, the tibial branch of the sciatic nerves were compared at the light and electron microscopic level to examine myelin thickness and total numbers of myelinated and unmyelinated fibres in normal and $VPAC_2R^{(-/-)}$ mice. The effects of selective antagonists and agonists of the $VPAC_2$ receptor were examined on reflex withdrawal responses to noxious heat and innocuous mechanical stimulation.

Baseline reflex withdrawal responses to noxious heat and innocuous mechanical stimulation were measured in normal and $VPAC_2R^{(-/-)}$ mice and measurements were made until day 21 post surgery. An antagonist of the $VPAC_2$ receptor (Des-1-4-Arg^{16}-Ro25-1553) was administered intrathecally in conscious mice 2-3 weeks following CCI surgery in normal and $VPAC_2R^{(-/-)}$ mice and reflex withdrawal responses to heat and mechanical stimuli were recorded. An agonist of the $VPAC_2$ receptor (Ro25-1553) was also administered intrathecally in normal and $VPAC_2R^{(-/-)}$ mice 2-3 weeks following CCI and reflex withdrawal responses to heat and mechanical stimuli were recorded. Injections were carried out in wild-type litter mate $VPAC_2R^{(+/+)}$ mice for control purposes. The general methods are described in detail in chapter 2.

3.7 Results

3.7.1 Behavioural Analysis of Sensory Responses in Wild-Type and $VPAC_2$ Receptor$^{(-/-)}$ Mice Following CCI

These results were obtained from a total of 38 mice (n=28 CCI and n=10 sham-operated). Throughout these experiments the experimenter was blinded to the genotype of the subject to eliminate bias and an independent third party was responsible for identification of mice after the experiments were terminated on completion. Reflex withdrawal responses to noxious heat (Hargreaves test) and normally innocuous mechanical stimuli (von Frey filaments) were measured. Several days of pre-operative baseline testing was carried out in order to establish a stable baseline for comparison to post-operative values, and following CCI surgery the behavioural testing recommenced at day 3 post-operatively. Behavioural testing continued daily for a minimum period of four weeks and / or until reflex withdrawal response values had returned to baseline values.

Following CCI surgery in wild-type mice, there was a characteristic and significant decrease in the ipsilateral paw withdrawal latency to noxious heat (Figure 3.2A). There was also a similar decrease in the paw withdrawal threshold to previously innocuous mechanical stimulation using von Frey filaments and these abnormal behavioural responses peaked at around 10-14 days post-operatively (Figure 3.3A). In contrast, the characteristic development of abnormal behavioural reflex responses to noxious heat and innocuous mechanical stimuli following CCI were delayed and attenuated in $VPAC_2R^{(-/-)}$ mice. Although there was still a significant decrease in the paw withdrawal latency/threshold to noxious heat and previously innocuous mechanical stimuli as in wild-type littermates (Figure 3.2B and 3.3B), both the magnitude and duration of the changes were dramatically attenuated. Sham surgery in wild-type mice was carried out in parallel with CCI surgery to demonstrate that the effects were not simply due to any of the surgical procedures. Following sham-surgery there were no characteristic decreases in the reflex withdrawal responses to noxious heat or mechanical stimuli (Figure 3.2 C and 3.3 C) respectively.

Figure 3.2 Analysis of Paw Withdrawal Latency to Noxious Heat in Wild-Type and $VPAC_2$ Receptor$^{(-/-)}$ Mice Following CCI or Sham Surgery

Data are presented as mean paw withdrawal latency (s) from noxious heat stimuli for ipsilateral and contralateral paws plotted against days pre- and post-surgery.

(A) In wild-type littermates of the mutant mice, paw withdrawal latency to noxious heat ipsilateral to nerve injury was significantly reduced following CCI, when compared to pre-operative baseline values († $p \leq 0.05$; one way ANOVA followed by Neuman-Keuls post-hoc test) and from postoperative contralateral values (* $p \leq 0.05$; Student's paired t-test).

(B) In $VPAC_2$ receptor$^{(-/-)}$ mice, paw withdrawal latency to noxious heat ipsilateral to nerve injury was significantly reduced following CCI when compared to baseline values († $p \leq 0.05$; one way ANOVA followed by Neuman-Keuls post-hoc test) and from post-operative contralateral values (* $p \leq 0.05$; Student's paired t-test). However, the duration and extent was markedly diminished when compared to wild-type mice.

(C) In wild-type littermates of the mutant mice, paw withdrawal latency to noxious heat was unaffected (one way ANOVA) following sham surgery, which was carried out for control purposes.

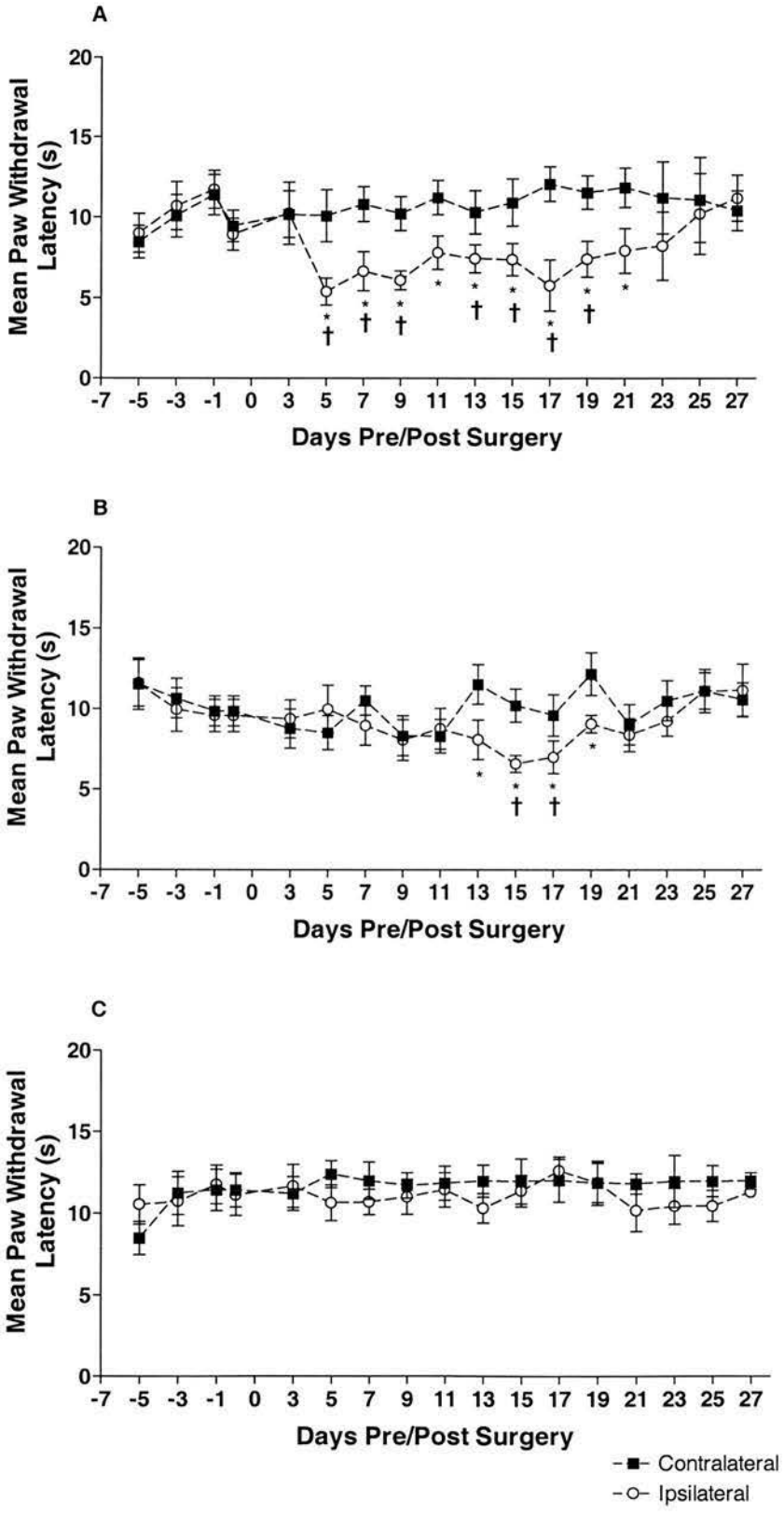

A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-7 -5 -3 -1 0 3 5 7 9 11 13 15 17 19 21 23 25 27
Days Pre/Post Surgery
B
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-7 -5 -3 -1 0 3 5 7 9 11 13 15 17 19 21 23 25 27
Days Pre/Post Surgery
C
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-7 -5 -3 -1 0 3 5 7 9 11 13 15 17 19 21 23 25 27
Days Pre/Post Surgery
Contralateral
Ipsilateral

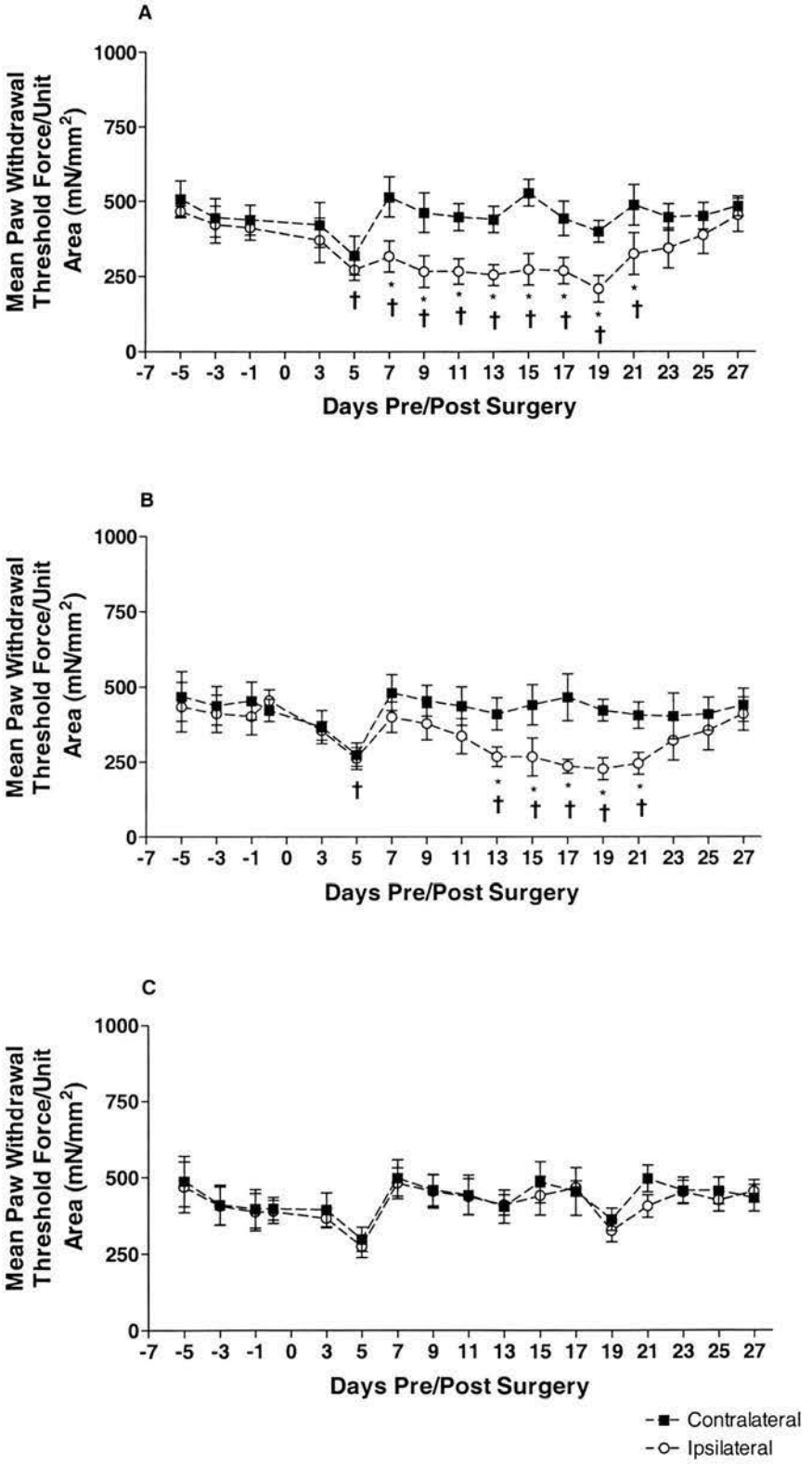
Figure 3.3 Analysis of Reflex Withdrawal Responses to Innocuous Mechanical Stimuli in Wild-Type and $VPAC_2$ Receptor$^{(-/-)}$ Mice Following CCI or Sham Surgery

Data are presented as mean paw withdrawal threshold (mN/mm^2) to innocuous mechanical stimuli for ipsilateral and contralateral paws plotted against time (days) pre- and post-surgery.

(A) In wild-type littermates of the mutant mice, paw withdrawal threshold to normally innocuous mechanical stimuli ipsilateral to nerve injury was significantly decreased following CCI, when compared to pre-operative values († $p \leq 0.05$; Kruskal-Wallis ANOVA followed by Dunn's post-hoc test) and from post-operative contralateral values (* $p \leq 0.05$; Mann-Whitney U test).

(B) In $VPAC_2$ receptor$^{(-/-)}$ mice, paw withdrawal threshold to normally innocuous mechanical stimuli ipsilateral to nerve injury was significantly decreased following CCI when compared to pre-operative values († $p \leq 0.05$; Kruskal-Wallis ANOVA followed by a Dunn's post-hoc test) and from post-operative contralateral values (* $p \leq 0.05$; Mann-Whitney U test). However, the duration and extent was markedly diminished when compared to wild-type mice.

(C) In wild-type littermate mice, paw withdrawal threshold to normally innocuous mechanical stimuli was unaffected (Kruskal-Wallis ANOVA) following sham surgery, which was carried out for control purposes.


A
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm²)
1000
750
500
250
0
-7 -5 -3 -1 0 3 5 7 9 11 13 15 17 19 21 23 25 27
Days Pre/Post Surgery
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm²)
1000
750
500
250
0
-7 -5 -3 -1 0 3 5 7 9 11 13 15 17 19 21 23 25 27
Days Pre/Post Surgery
C
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm²)
1000
750
500
250
0
-7 -5 -3 -1 0 3 5 7 9 11 13 15 17 19 21 23 25 27
Days Pre/Post Surgery
Contralateral
Ipsilateral

3.7.2 Sciatic Nerve Morphology

To exclude the possibility that any behavioural changes were associated with developmental abnormalities in the pattern of myelination in sciatic afferents, axon diameter and myelin thickness profiles were examined by light and electron microscopy. In unoperated $VPAC_2R^{(-/-)}$ mice and wild-type mice the sciatic nerves were examined by light microscopy to determine if the myelin sheath was affected. In all experiments the experimenter was blinded to the genotype to eliminate bias and in all mice tested there was no overt alteration in the gross appearance of peripheral nerves of $VPAC_2R^{(-/-)}$ mice when compared to wild-type. Myelin thickness was measured by measuring axon diameter / external diameter and plotting G-ratios (Figure 3.4). There was no statistically significant alteration between $VPAC_2R^{(-/-)}$ mice and wild-type littermates, and the myelinated fibres of $VPAC_2R^{(-/-)}$ mice appeared structurally normal. Electron microscopy was carried out to assess axon diameter of unmyelinated fibres and to count total numbers of myelinated and unmyelinated fibres in wild-type and $VPAC_2R^{(-/-)}$ mice. In all samples both axon diameter and myelin thickness profiles for myelinated (Aδ and Aβ) fibres and the diameters of unmyelinated (C) fibres were similar between unoperated wild-type and $VPAC_2R^{(-/-)}$ mice (Figure 3.5, 3.6). Values were in accordance with previous reports (Guilbaud et al., 1993; Sommer et al., 1995).

Figure 3.4 Morphological Analysis of Myelin Sheath Thickness of Myelinated Fibres Located in the Tibial Branch of the Sciatic Nerve of Normal Wild-Type and $VPAC_2$ Receptor$^{(-/-)}$ Mice

(A) Myelin sheath thickness of myelinated fibres in terms of G-ratio (internal / external diameter) as a function of the external diameter in normal wild-type mice (littermates to the mutant animals, n=3). In each nerve 500 fibres were used for measurement of myelin thickness.

(B) Myelin sheath thickness of myelinated fibres in terms of G-ratio (internal / external diameter) as a function of the external diameter in $VPAC_2R^{(-/-)}$ mice (n=3). In each nerve 500 fibres were used for measurement of myelin thickness.

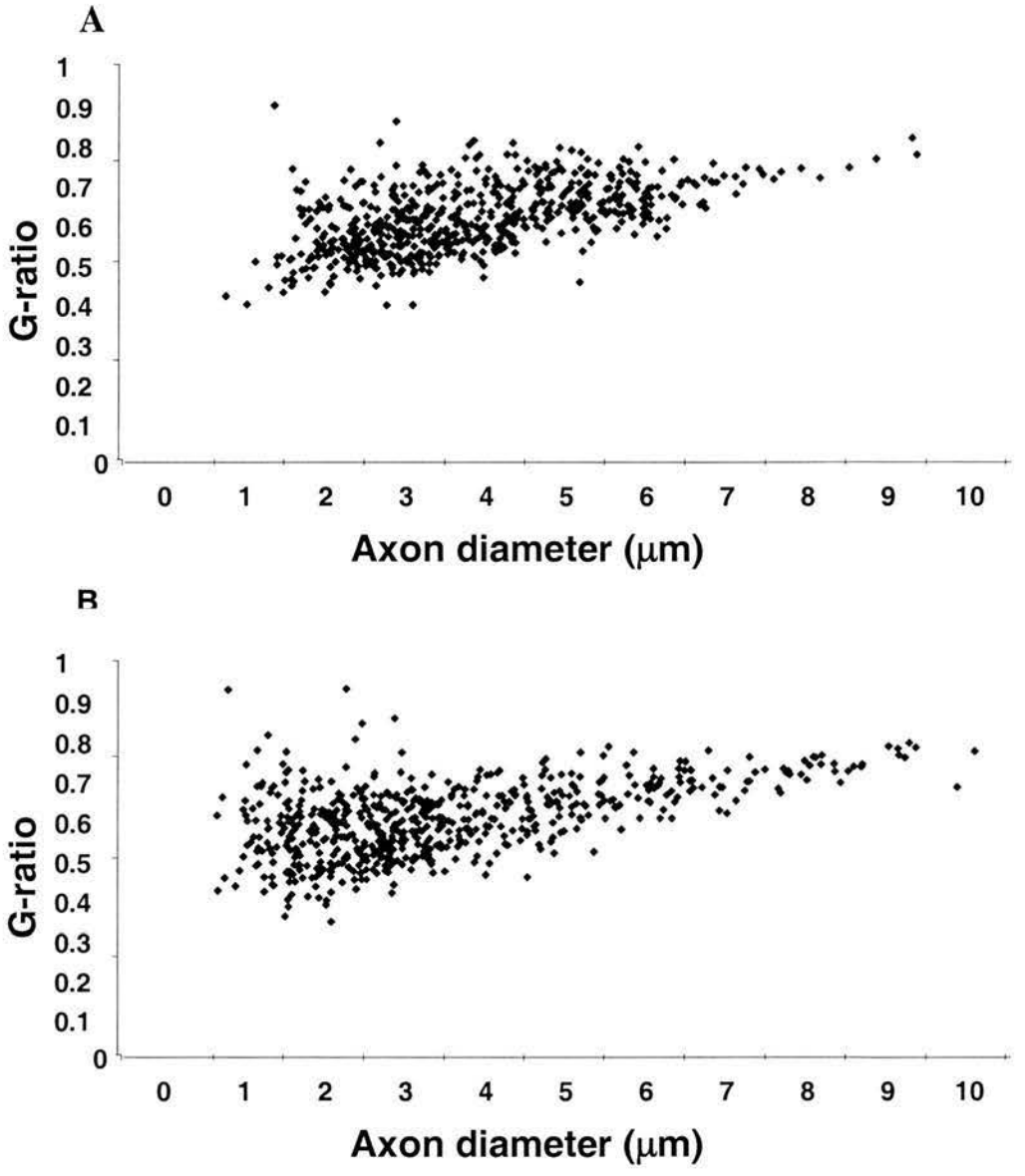

A
G-ratio
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7 8 9 10
Axon diameter (μm)
B
G-ratio
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7 8 9 10
Axon diameter (μm)

Figure 3.5 Morphological Analysis of Axon Diameters of Myelinated Fibres Located in the Tibial Branch of the Sciatic Nerve of Normal Wild-Type and $VPAC_2$ Receptor$^{(-/-)}$ Mice

(A) Frequency distribution of the axon diameters of myelinated fibres in the Aδ/Aβ diameter range from the tibial branch of the sciatic nerve in unoperated wild-type mice (littermates of the mutant animals, n=3). In each nerve 750 fibres were used for diameter measurement.

(B) Frequency distribution of the axon diameters of myelinated fibres in the Aδ/Aβ diameter range from the tibial branch of the sciatic nerve in unoperated $VPAC_2R^{(-/-)}$ mice. In each nerve 750 fibres were used for diameter measurement.

No significance differences were observed (χ^2 test) at the light microscopy level for myelinated A fibre diameter distributions between wild-type and $VPAC_2R^{(-/-)}$ mutant mice.

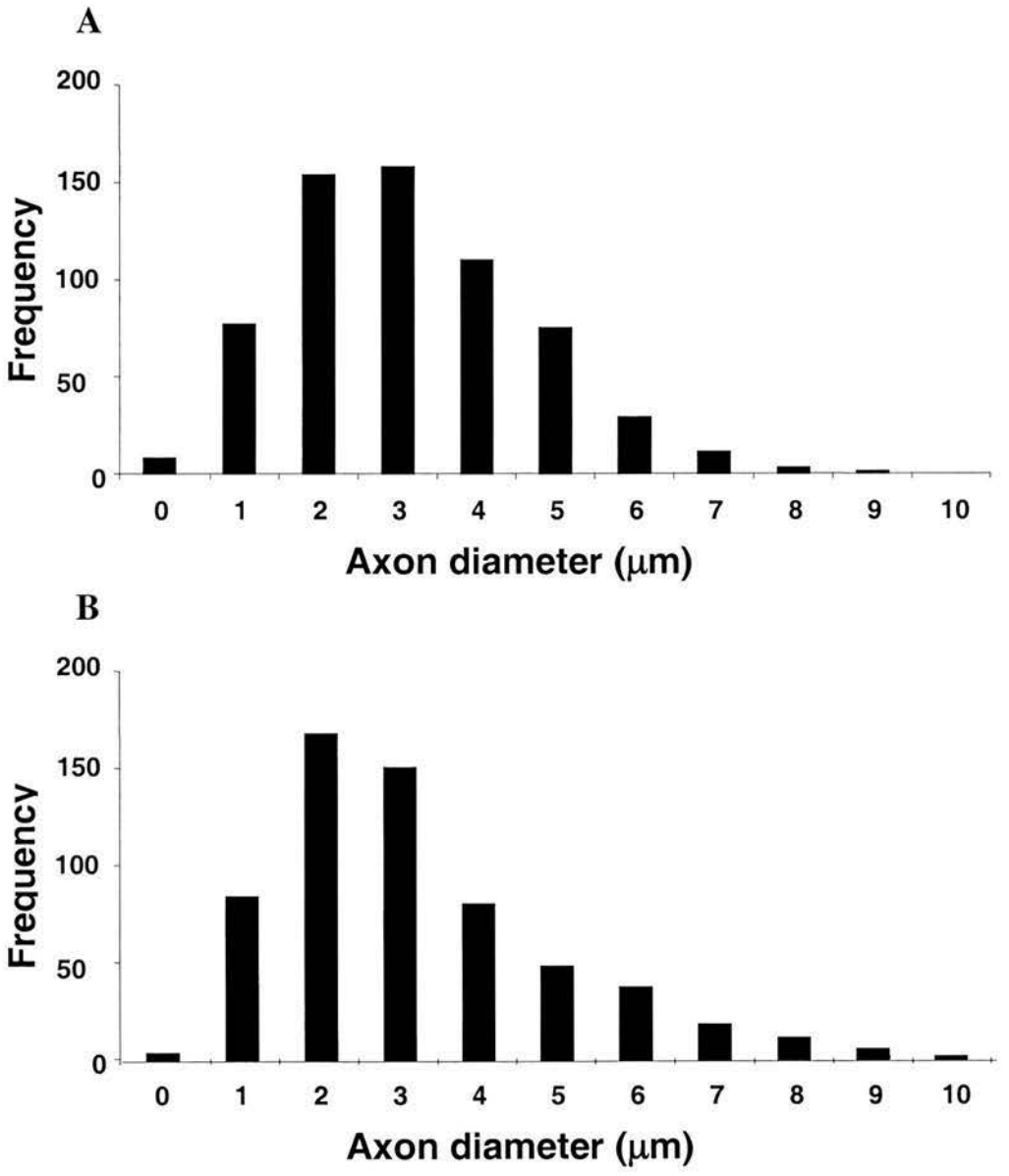
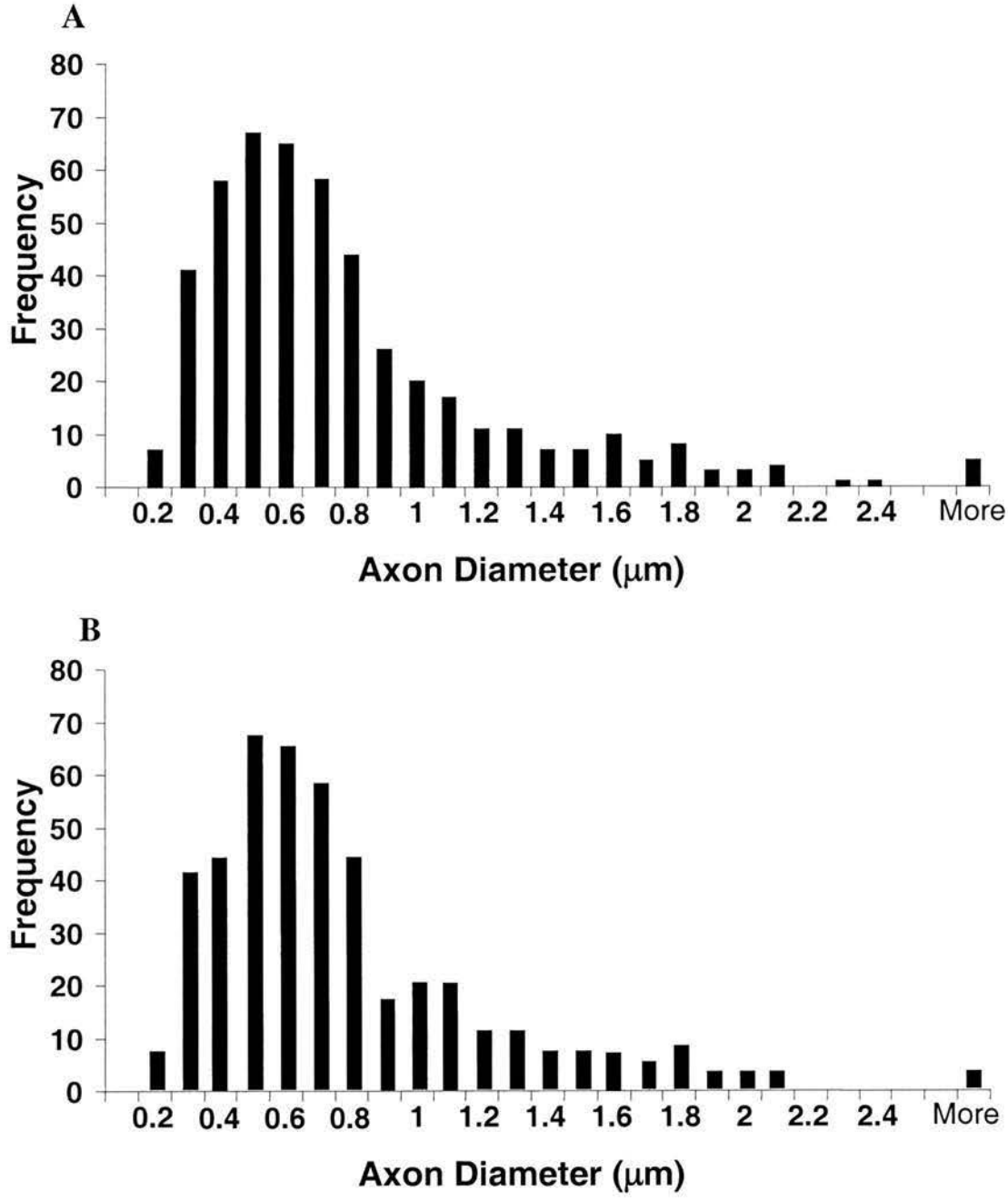

A
200
150
100
50
0
Frequency
0 1 2 3 4 5 6 7 8 9 10
Axon diameter (μm)
B
200
150
100
50
0
Frequency
0 1 2 3 4 5 6 7 8 9 10
Axon diameter (μm)

Figure 3.6 Morphological Analysis of Axon Diameters of Unmyelinated Fibres in the Tibial Branch of the Sciatic Nerve of Normal Wild-Type and $VPAC_2$ Receptor$^{(-/-)}$ Mice

(A) Frequency distribution of the axon diameters of unmyelinated C-fibres from the tibial branch of the sciatic nerve in wild-type mice (littermates of the mutant mice, n=2). In each nerve 250 fibres were used for diameter measurement.

(B) Frequency distribution of the axon diameters of unmyelinated C-fibres from the tibial branch of the sciatic nerve in $VPAC_2R^{(-/-)}$ mice. In each nerve 250 fibres were used for diameter measurement.

No significance differences were observed (χ^2 test) at the electron microscopy level for unmyelinated C-fibre diameter distributions between wild-type and $VPAC_2R^{(-/-)}$ mutant mice.


A
80
70
60
50
40
30
20
10
0
Frequency
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
2.2
2.4
More
Axon Diameter (μm)
B
80
70
60
50
40
30
20
10
0
Frequency
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
2.2
2.4
More
Axon Diameter (μm)

3.7.3 Effects of Intrathecal Administration of the Highly Selective $VPAC_2R$ Antagonist des (1-4) Arg^{16}-Ro 25-1553 on Reflex Withdrawal Responses to Noxious Heat and Innocuous Mechanical Stimuli in Wild-Type and $VPAC_2$ $Receptor^{(-/-)}$ Mice Following CCI

Results were obtained from a total of 28 mice, (n=14 wild-type littermates of the mutant animals and n=14 $VPAC_2$ $receptor^{(-/-)}$). Intrathecal administration of the selective $VPAC_2R$ antagonist des (1-4) Arg^{16}-Ro 25-1553 was carried out as described previously (Chapter 2 section 2.3.4).

In wild-type mice exhibiting peak behavioural changes following CCI, des-(1-4)-Arg^{16}-Ro 25-1553 significantly reversed the increased reflex withdrawal responses to noxious heat and mechanical stimulation (Figure 3.7 A and B). This inhibition was monitored over a 90 minute testing period. In $VPAC_2R^{(-/-)}$ mice exhibiting peak behavioural changes following CCI, des-(1-4)-Arg^{16}-Ro 25-1553 had no significant effect on the increased reflex withdrawal responses to noxious heat and mechanical stimulation (Figure 3.8 A and B). Control intrathecal administration of vehicle in wild-type CCI mice had no significant effect on reflex withdrawal responses to noxious heat and normally innocuous mechanical stimuli (Figure 3.11).

Figure 3.7 Effects of Intrathecal Administration of the $VPAC_2$ Receptor Antagonist des-(1-4) Arg^{16}-Ro 25-1553 on Reflex Withdrawal Responses to Noxious Heat and Innocuous Mechanical Stimuli in Wild-Type Mice Following CCI

Data are presented as mean paw withdrawal latency (s) from noxious heat (A), and mean paw withdrawal threshold (mN/mm^2) to innocuous mechanical stimuli (B) for ipsilateral and contralateral paws plotted against time (min) pre-and post-injection (n=7). Arrows mark intrathecal drug administration.

(A) In wild-type mice (littermates to mutant animals) exhibiting peak behavioural changes following CCI, paw withdrawal latency to noxious heat stimuli ipsilateral (but not contralateral) showed significant differences between pre- and post-drug administration values († $p \leq 0.05$; one way ANOVA followed by Neuman-Keuls post-hoc test). Significant differences between ipsilateral and contralateral paw withdrawal latencies are indicated (* $p \leq 0.05$; Student's paired t-test). des-(1-4)-Arg^{16}-Ro 25-1553 was administered at a dose of [200pmol in 20µl].

(B) In wild-type mice exhibiting peak behavioural changes following CCI, paw withdrawal threshold to innocuous mechanical stimuli ipsilateral (but not contralateral) to nerve injury showed significant differences between pre-and post-drug administration values († $p \leq 0.05$; Kruskal-Wallis ANOVA followed by Dunn's post-hoc test). Significant differences between ipsilateral and contralateral withdrawal thresholds are indicated (* $p \leq 0.05$; Mann-Whitney U test). des-(1-4)-Arg^{16}-Ro25-1553 was administered at a dose of [200pmol in 20µl].

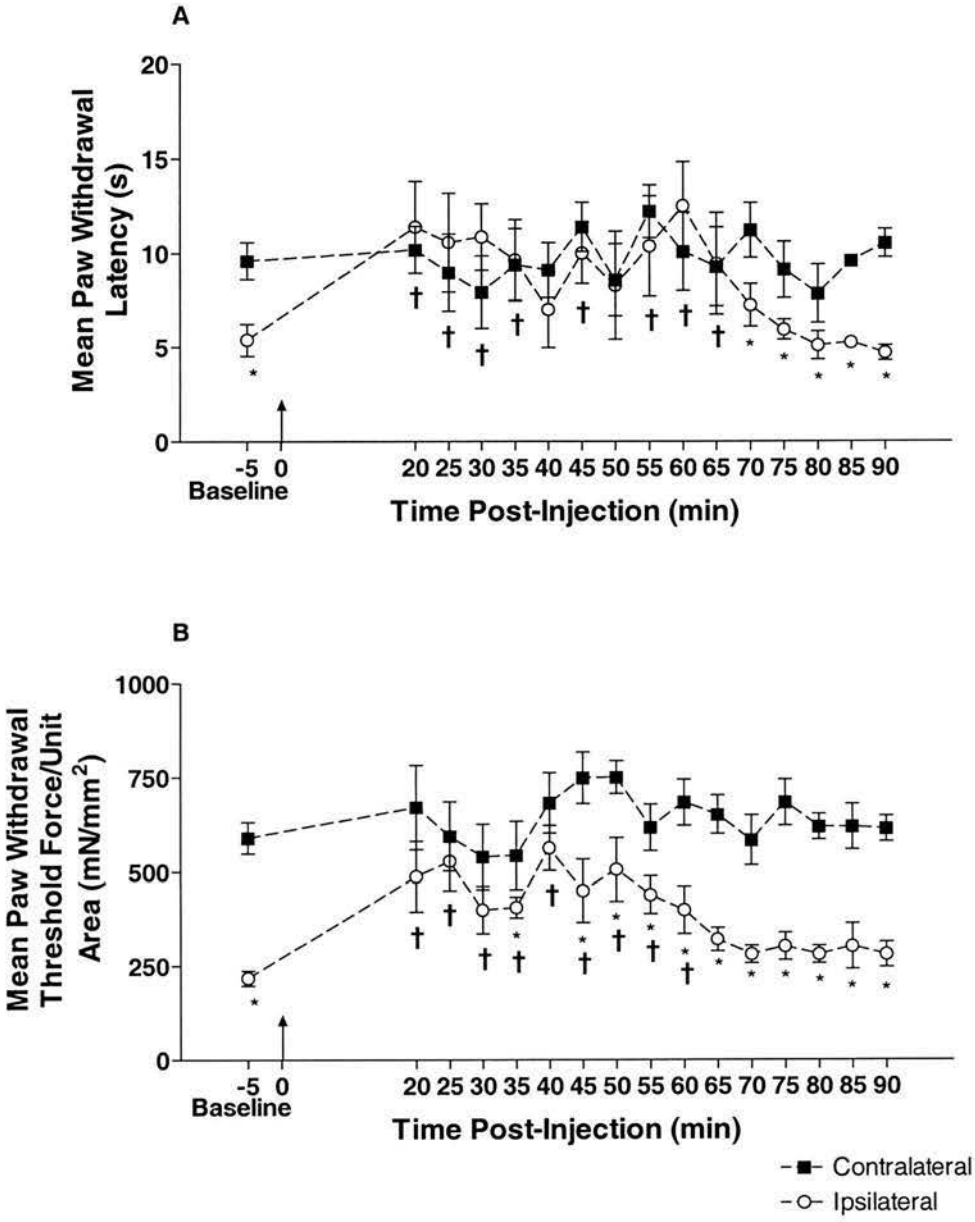
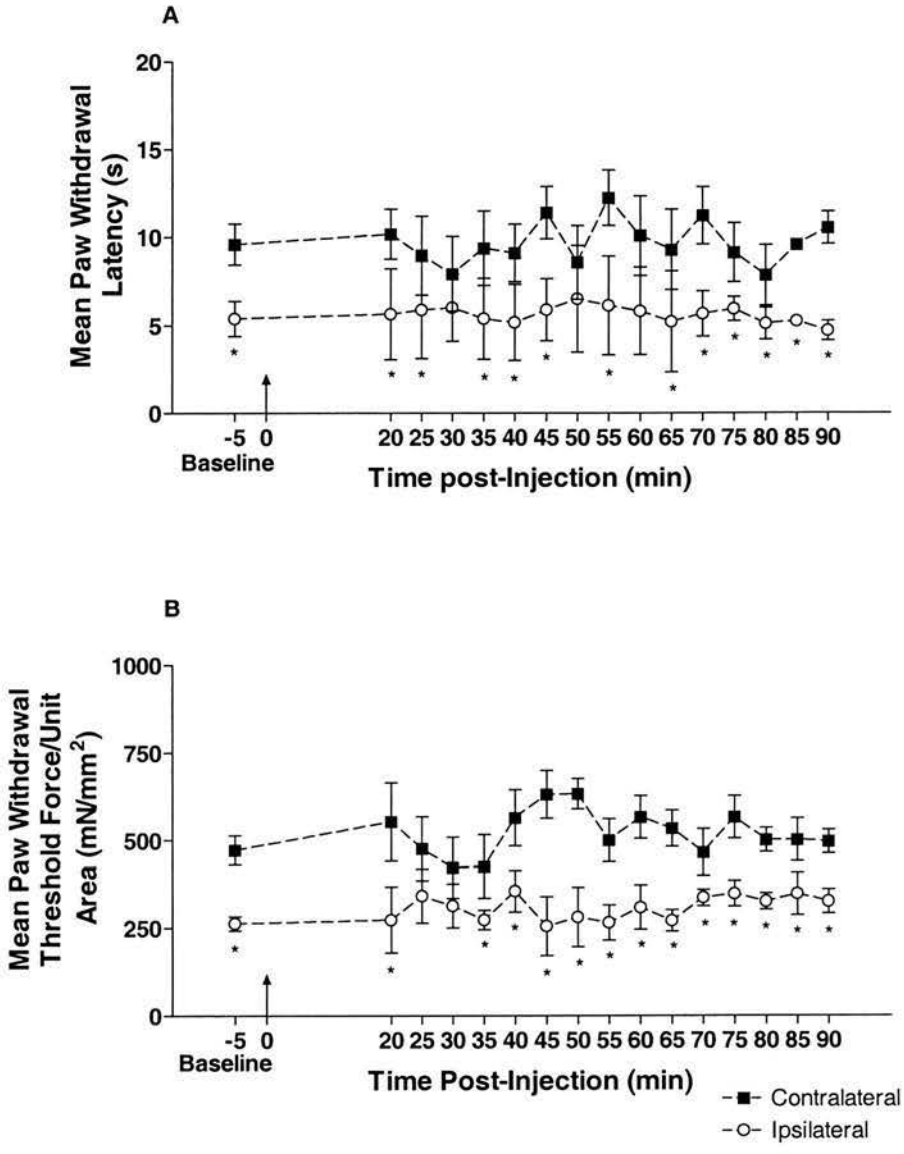

A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm2)
1000
750
500
250
0
-5 0
Baseline
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
Contralateral
Ipsilateral

Figure 3.8 Effects of Intrathecal Administration of the $VPAC_2R$ Antagonist des-(1-4) Arg^{16}-Ro 25-1553 on Reflex Withdrawal Responses to Noxious Heat and Innocuous Mechanical Stimuli in $VPAC_2$ $Receptor^{(-/-)}$ Mice Following CCI

Data are presented as mean paw withdrawal latency (s) from noxious heat (A), and mean paw withdrawal threshold (mN/mm^2) to innocuous mechanical stimuli (B) for ipsilateral and contralateral paws plotted against time (min) pre-and post-injection (n=7). Arrow marks intrathecal drug administration.

(A) In $VPAC_2R^{(-/-)}$ mice exhibiting peak behavioural changes following CCI, paw withdrawal latency to noxious heat ipsilateral (but not contralateral) to nerve injury showed significant differences between pre-and post-drug administration values († $p \leq 0.05$; one way ANOVA followed by Neuman-Keuls post-hoc test). Significant differences between contralateral and ipsilateral values are indicated (* $p \leq 0.05$; Student's paired t-test). des-(1-4)-Arg^{16}-Ro 25-1553 was administered at a dose of [200pmol in 20µl].

(B) In $VPAC_2R^{(-/-)}$ mice exhibiting peak behavioural changes following CCI, paw withdrawal threshold to innocuous mechanical stimuli ipsilateral (but not contralateral) to nerve injury showed significant differences between pre- and post-drug administration values values († $p \leq 0.05$; Kruskal-Wallis ANOVA followed by Dunn's post-hoc test). Significant differences between contralateral and ipsilateral values are indicated (* $p \leq 0.05$; Mann-Whitney U test). des-(1-4)-Arg^{16}-Ro 25-1553 was administered at a dose of [200pmol in 20µl].


A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm2)
1000
750
500
250
0
-5 0
Baseline
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
Contralateral
Ipsilateral

3.7.4 Effects of Intrathecal Administration of the Selective $VPAC_2$ Receptor Agonist Ro 25-1553 on Sensory Reflex Withdrawal Responses in Wild-Type and $VPAC_2R^{(-/-)}$ Mice

Results were obtained from a total of 16 mice, (n=8 wild-type littermates of the mutant animals and n=8 $VPAC_2$ receptor$^{(-/-)}$). Intrathecal administration of the selective $VPAC_2R$ agonist Ro 25-1553 was carried out as described previously (chapter 2 section 2.3.4). In unoperated wild-type mice, the $VPAC_2R$ agonist Ro 25-1553 significantly decreased the latency and threshold respectively of reflex withdrawal responses to noxious heat and mechanical stimulation compared to pre-injection values (Figure 3.9 A and B). Following drug administration, responses were monitored over a 90 minute testing period during which recovery was observed. In $VPAC_2R^{(-/-)}$ mice the $VPAC_2R$ agonist Ro 25-1553 had no significant effect on the reflex withdrawal responses to noxious heat and innocuous mechanical stimulation (Figure 3.10 A and B). Testing continued for 90 minutes. Control intrathecal administration of saline in wild-type CCI mice had no significant effect on reflex withdrawal responses to noxious heat and innocuous mechanical stimuli (Figure 3.11).

Figure 3.9 Effects of Intrathecal Administration of the $VPAC_2$ Receptor Agonist Ro 25-1553 on Reflex Withdrawal Responses to Noxious Heat and Innocuous Mechanical Stimuli in Normal Wild-Type Mice

Data are presented as mean paw withdrawal latency (s) from noxious heat (A), and mean paw withdrawal threshold (mN/mm^2) to innocuous mechanical stimuli (B) plotted against time (min) pre-and post-injection (n=8). Arrow marks intrathecal drug administration.

(A) In normal wild-type mice (littermates of mutant mice) mean paw withdrawal latency from noxious heat stimuli showed significant differences between pre-and post-drug administration values (* $p \leq 0.05$; one way ANOVA followed by Neuman-Keuls post-hoc test). Ro 25-1553 was administered at a dose of [200pmol in 20µl].

(B) In normal wild-type mice (littermates of mutant mice) mean paw withdrawal threshold to innocuous mechanical stimuli showed significant differences between pre-and post- drug administration values (* $p \leq 0.05$; Kruskal-Wallis ANOVA followed by Dunn's post-hoc test). Ro 25-1553 was administered at a dose of [200pmol in 20µl].

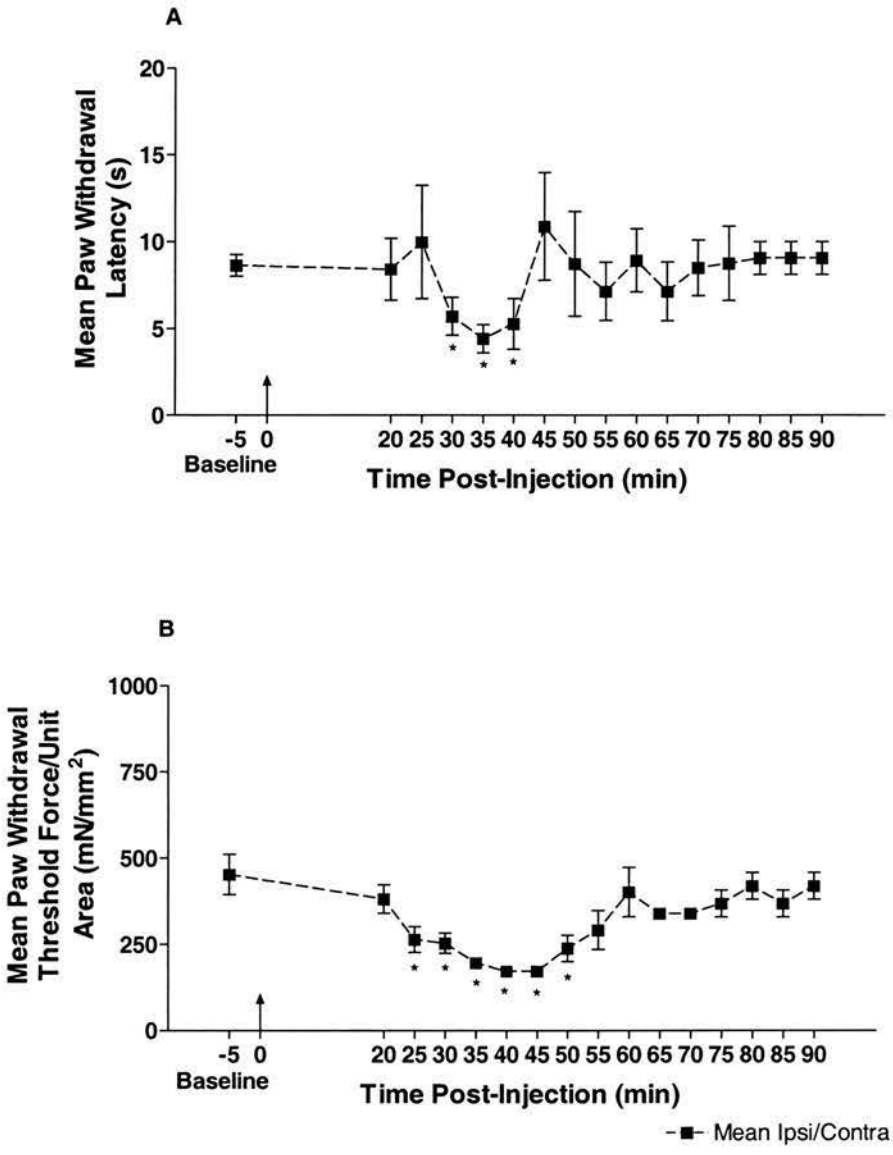

A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm2)
1000
750
500
250
0
-5 0
Baseline
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
Mean Ipsi/Contra

Figure 3.10 Effects of Intrathecal Administration of the $VPAC_2$ Receptor Agonist Ro 25-1553 on Reflex Withdrawal Responses to Noxious Heat and Innocuous Mechanical Stimuli in $VPAC_2$ Receptor$^{(-/-)}$

Data are presented as mean paw withdrawal latency (s) from noxious heat (A), and mean paw withdrawal threshold (mN/mm^2) to innocuous mechanical stimuli (B) plotted against time (min) pre- and post-injection (n=8). Arrow marks intrathecal drug administration.

(A) In normal $VPAC_2R^{(-/-)}$ mean paw withdrawal latency to noxious heat showed no significant difference between pre-and post-drug administration values (one way ANOVA). Ro 25-1553 was administered at a dose of [200pmol in 20µl].

(B) In $VPAC_2R^{(-/-)}$ mice exhibiting peak behavioural changes following CCI, mean paw withdrawal threshold to innocuous mechanical stimulation showed no significant difference between pre- and post-drug administration values (Kruskal-Wallis ANOVA). Ro 25-1553 was administered at a dose of [200pmol in 20µl].

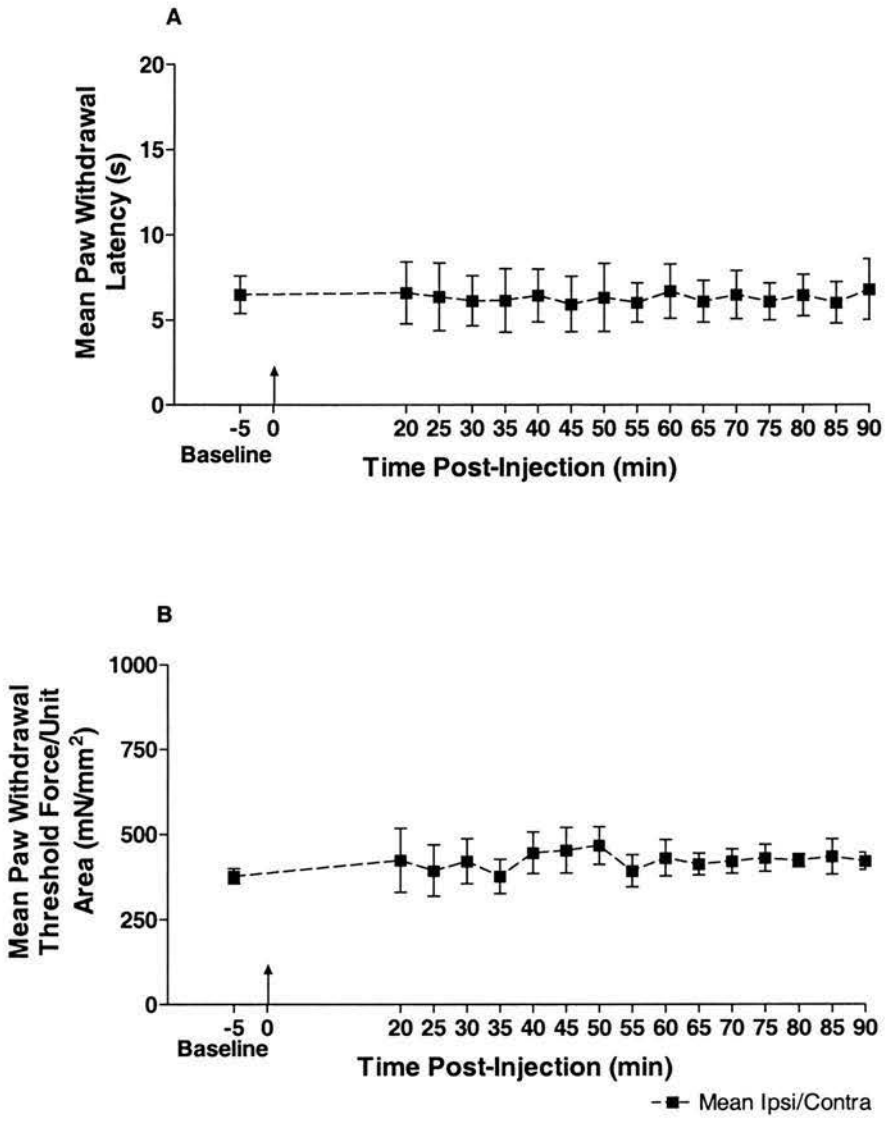
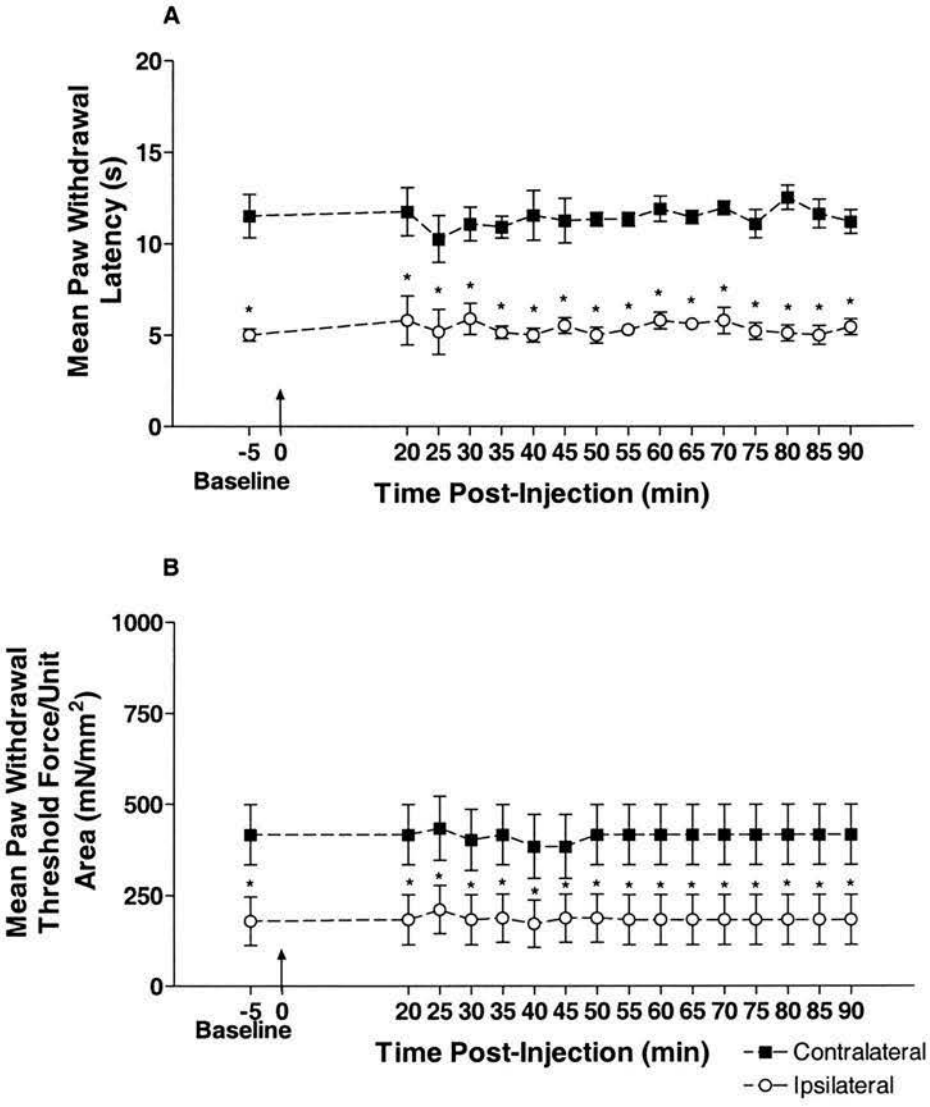

A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm2)
1000
750
500
250
0
-5 0
Baseline
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
Mean Ipsi/Contra

Figure 3.11 Effects of Intrathecal Administration of Vehicle on Reflex Withdrawal Responses to Noxious Heat and Innocuous Mechanical Stimuli in Wild-Type Mice Following CCI

Data are presented as mean paw withdrawal latency (s) from noxious heat (A), and mean paw withdrawal threshold (mN/mm^2) to innocuous mechanical stimuli (B) for ipsilateral and contralateral paws plotted against time (min) pre- and post-injection (n=6). Arrow marks intrathecal drug administration.

(A) In wild-type mice exhibiting peak behavioural changes following CCI, paw withdrawal latency to noxious stimuli ipsilateral to nerve injury showed no significant difference between pre-and post-vehicle administration values (one way ANOVA). Significant differences between contralateral and ipsilateral values are indicated (* $p \leq 0.05$, Student's paired t-test). Vehicle was administered at a volume of 20µl.

(B) In wild-type mice exhibiting peak behavioural changes following CCI, paw withdrawal threshold to innocuous mechanical stimuli ipsilateral to nerve injury showed no significant difference between pre-and post-vehicle administration values (Kruskal-Wallis ANOVA). Significant differences between contralateral and ipsilateral values are indicated (* $p \leq 0.05$, Mann-Whitney U test). Vehicle was administered at a volume of 20µl.


A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm2)
1000
750
500
250
0
-5 0
Baseline
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
Contralateral
Ipsilateral

3.8 Discussion

VIP and the two alternatively processed forms of the PACAP precursor (PACAP-27 and PACAP-38) are recognised by a family of three G protein coupled receptors (Harmer and Lutz, 1994), namely the PACAP receptor (Shivers et al., 1991; Hashimoto et al., 1993), the $VPAC_1$ receptor (Ishihara et al., 1992) and the $VPAC_2$ receptor (Lutz et al., 1993). There is a distinct distribution of the $VPAC_1$, $VPAC_2$ and PACAP receptors in both the peripheral and central nervous systems of the rat (Harmer and Lutz, 1994; Lutz et al., 1993, Shioda et al., 1997, Usdin et al., 1994; Vertongen et al., 1997; Dickinson et al., 1999). Binding sites for VIP and PACAP ligands have been shown to be present in the rat spinal cord, particularly in the lumbar and sacral segments, with the highest concentrations in lamina I and II of the dorsal horn (Kar and Quirion, 1995; Moller et al., 1993; Yashpal et al., 1991). The mRNA for $VPAC_1$ (Ishihara et al., 1992), $VPAC_2$ (Sheward et al., 1995) and PACAP receptors (Arimura and Shioda, 1995) is also expressed in the spinal dorsal horn.

Following peripheral nerve injury, a number of neurochemical changes within DRG neurones and the spinal dorsal horn have been well documented including a marked up-regulation of the expression of VIP and PACAP (Hokfelt et al., 1994). Previous evidence has suggested that these peptides may have neurotransmitter and / or neuromodulatory functions within the CNS (Jeftinija et al., 1982; Phillis et al., 1978; Narita et al., 1996; Salt and Hill, 1981; Xu and Weisenfeld-Hallin, 1996). In fact following CCI there is a marked increase in the levels of mRNA for the $VPAC_2$ receptor rather than $VPAC_1$ or PACAP receptors (Dickinson et al., 1999) and concomitant functional data data suggest that the $VPAC_2$ receptor may play an important role in the transmission of sensory information within the spinal cord of both normal mice and especially in neuropathic mice (Dickinson et al., 1997; 1999).

CCI was carried out in $VPAC_2R^{(-/-)}$ mice to evaluate the contribution of the $VPAC_2$ receptor to the development and maintenance of the abnormal behavioural responses that develop following CCI. CCI was also carried out in wild-type littermate mice for comparison. Following surgery, the wild-type mice developed the abnormal ipsilateral behavioural responses indicative of neuropathic pain. However, in mice

lacking the $VPAC_2$ receptor, the development of mechanical allodynia and thermal hyperalgesia was markedly reduced. The magnitude of ipsilateral, injury-induced decrease in paw withdrawal latency to noxious heat, also appeared to be diminished in mutant compared to wild-type mice. Effects on the magnitude of change in paw withdrawal threshold to normally innocuous stimuli were however less. Sham surgery was carried out for control purposes and no alteration in reflex withdrawal responses from noxious heat or innocuous mechanical stimuli were observed at any time point. These results suggest that the $VPAC_2$ receptor may play a key role in the mechanisms underlying the development and maintenance of the abnormal behavioural responses that follow CCI believed to be indicative of neuropathic pain. In support of this is the observation that the levels of immunoreactive VIP are markedly increased from almost undetectable levels in the dorsal horn, to high levels following nerve injury (Hokfelt et al., 1994), and the increase in the levels of mRNA for the $VPAC_2$ receptor in the ipsilateral dorsal horn following CCI (Dickinson et al., 1999). These results taken together suggest that there may be an increased involvement of the $VPAC_2$ receptor in spinal sensory processing following nerve injury that may participate in the development and maintenance of neuropathic pain.

Intrathecal administration of the $VPAC_2$ receptor inhibitor (Des-1-4-Arg^{16}-Ro 25-1553) was carried out to further evaluate the contribution of the $VPAC_2$ receptor to the maintenance of the abnormal behavioural responses that occur in established CCI neuropathy. In wild-type mice at the peak of neuropathic reflex changes, intrathecal injection of Des-1-4-Arg^{16}-Ro 25-1553 significantly reversed the injury-induced changes in ipsilateral reflex withdrawal responses. This reversal was specific to the sensitised responses from the ipsilateral limb and the antagonist showed no effect on responses from the contralateral limb. Control intrathecal administration of saline in wild-type mice had no effect. Intrathecal administration of Des-1-4-Arg^{16}-Ro 25-1553 in $VPAC_2R^{(-/-)}$ mice was carried out to further confirm both the specificity of the drug and the genotype of the $VPAC_2R^{(-/-)}$ mice and the antagonist had no effect on reflex responses. The antinociceptive effect of the novel $VPAC_2$ receptor antagonist following CCI in wild-type mice was in agreement with previous electrophysiological studies which applied the $VPAC_2$ receptor antagonist in the

proximity of single dorsal horn neurones whose activity was evoked by the application of mustard-oil. The effects of Des-1-4-Arg^{16}-Ro 25-1553 were selectively antinociceptive, (markedly inhibiting the sustained C-fibre activity induced by topical application of the chemical irritant mustard oil), (Dickinson et al., 1997; 1999). The inhibitor also inhibited mustard oil-induced activity in nerve injured animals (Dickinson et al., 1999). These results taken together suggest that inhibitors of the $VPAC_2$ receptor may serve as selective analgesics, strongly reducing polymodal C-fibre mediated pain in normal and neuropathic pain states. In the present behavioural studies, the attenuating effects of the $VPAC_2$ receptor antagonist and of the $VPAC_2$ receptor knockout were more marked on sensitised responses to heat than to mechanical stimuli. This suggests that $VPAC_2$ receptor antagonists might be useful to target selectively the hyperalgesia seen in relation to C-fibre nociceptor responses in neuropathic pain.

Previous findings have demonstrated that ionophoretic application of the $VPAC_2$ receptor agonist Ro 25-1553 in normal rats leads to a state of neuronal excitation which is further increased in nerve injured rats (Dickinson et al., 1999). To further assess the contribution of the $VPAC_2$ receptor and its potential modulatory role following nerve injury, parallel studies were carried out using intrathecal administration of the $VPAC_2$ receptor agonist Ro 25-1553 in both wild-type and $VPAC_2R^{(-/-)}$ mice that had undergone CCI. Intrathecal administration of Ro 25-1553 in wild-type mice dramatically decreased the paw withdrawal latency to noxious heat and the paw withdrawal threshold to innocuous mechanical stimuli with no effect in $VPAC_2R^{(-/-)}$ mice. These results provide further evidence that suggest that the $VPAC_2$ receptor may play a modulatory role in the processing of sensory information particularly following nerve injury.

Morphological investigation of the sciatic nerve was carried out in both wild-type and $VPAC_2R^{(-/-)}$ mice. Myelin thickness in A fibres and the frequency distribution of both A and C fibres were assessed to exclude the possibility that the differences observed for reflex withdrawal responses in the subsequent investigations were due to the presence / absence of the $VPAC_2$ receptor, and not due to any gross

developmental changes in the properties of the sciatic nerve. No structural changes in afferent fibres were detected.

It has been suggested using different experimental paradigms, that VIP and its action via the $VPAC_1$, $VPAC_2$ and PACAP receptors may in certain instances suppress or even prevent tissue responses to injury and consequent inflammation (reviewed in Said, 1998). Such evidence includes: (1) prevention of acute high-permeability pulmonary oedema caused by oxidant stress or glutamate; (2) attenuation of bronchoconstriction and airway inflammation induced by capsaicin, which releases the proinflammatory sensory neuropeptide tachykinins; (3) protection of CNS neurones against death induced by a variety of insults (Brenneman et al., 1998); and (4) inhibition of the production of tumour necrosis factor α and other inflammatory cytokines by lipopolysaccharide-stimulated macrophages (Delgado et al., 1999). However, whilst the mechanisms underpinning inflammatory and neuropathic pain states share a number of similarities, there are also a number of clear differences in the underlying mechanisms and it is perfectly reasonable to expect VIP, as a neuromodulator, to exert contrasting modulatory effects in the distinct mechanisms of inflammatory and neuropathic pain states. In the present study any potential influences of spinal VIP/PACAP receptors on inflammatory pain responses have not been investigated. In general, the anti-inflammatory actions reported for VIP are at peripheral tissue sites and often involve modifying the function of inflammation-related leukocytes. The facts that we are here assessing central rather than peripheral roles of these receptors and that VIP/PACAP expression in afferents is specifically up-regulated in the nerve injury model mean that any direct comparison between those experiments and the current study would not be possible.

In summary, these results provide further evidence of the modulatory role of VIP/PACAP within the dorsal horn of the spinal cord. This investigation also provides new insight into the role of the $VPAC_2$ receptor in the mechanisms underlying the development and maintenance of abnormal behavioural responses indicative of neuropathic pain following CCI. The combined evidence of this and recent studies indicates that the $VPAC_2$ receptor plays a key role in the thermal

hyperalgesia and mechanical allodynia following nerve injury and that antagonists of the $VPAC_2$ receptor may act as selective analgesics, strongly reducing polymodal-C-fibre-mediated pain in neuropathy. These novel findings also suggest that mechanical allodynia may be reversed in conscious mice following intrathecal administration of $VPAC_2$ receptor antagonists. The effects on mechanical allodynia appear less marked, but further extensive studies with a number of different agents would be needed to make a definitive conclusion this. Importantly, these experiments when added to previous findings underline the potential importance of the $VPAC_2$ receptor antagonists as new analgesics for use in currently intractable neuropathic pain states.

CHAPTER 4: THE ROLE OF CYCLIC-AMP DEPENDENT PROTEIN KINASE IN NEUROPATHIC PAIN

4.1. cAMP and the cAMP-Dependent Protein Kinase (PKA) Signalling System

Reversible protein phosphorylation is a key regulatory mechanism in eukaryotic cells. Protein phosphorylation was first demonstrated to regulate glycogen phosphorylase in response to glucagon (Fischer and Krebs, 1955; Sutherland and Wosilait, 1955) and the concept of 3', 5'-cyclic adenosine monophosphate (cAMP) was developed. The principal route of signal transduction by means of cAMP in mammalian cells, (with which the majority of biological effects of cAMP have been associated), is cAMP-dependent protein kinase (PKA) (Walsh et al., 1968). In the absence of cAMP, PKA is an enzymatically inactive tetrameric holoenzyme consisting of two homodimeric regulatory (R) subunits (RIα, RIβ, RIIα or RIIβ) and two catalytic (C) subunits (Cα and Cβ) (Krebs and Beavo., 1979; Nairn et al., 1985) (Figure 4.1). Activation of PKA occurs when four molecules of cAMP co-operatively bind to two sites on each R-subunit (Beebe et al., 1986). When both cAMP binding sites (A and B) are occupied, the R-subunit adopts a conformation with low affinity for the C-subunit, and the active C-subunits dissociate and phosphorylate serine / threonine residues within specific substrate proteins (Meinkoth et al., 1993). There is increasing evidence that an imbalance of the R:C-subunit equilibrium can lead to PKA becoming persistently activated at basal cAMP levels. This imbalance can be due in part to a degradation of the R-subunits by the ubiquitin proteasome pathway (Chain et al., 1999).

4.2. Isozymes of PKA

Two major types of PKA have been identified by DEAE ion exchange chromatography (Reimann et al., 1971; Corbin et al., 1975) and named type I and type II according to their elution order. The two types of holoenzyme differ in the structure of the regulatory subunit incorporated (RI or RII). Recent work has shown

that there are two RI subunits termed RIα (Lee et al., 1983; Sandberg et al., 1987) and RIβ (Clegg et al., 1988; Solberg et al., 1991) and two RII subunits termed RIIα and RIIβ (Scott et al., 1987; Oyen et al., 1989). Furthermore, two distinct C-subunits have been initially identified by molecular cloning, and designated Cα (Uhler et al., 1986) and Cβ (Uhler et al., 1986; Showers and Maurer, 1986). Low homology screening of Cα and Cβ-related sequences from human testis has also revealed an additional C-subunit designated Cγ (Beebe et al., 1990; Reinton et al., 1998). Splice variants of both Cα and Cβ have also been reported (Lange-Carter and Malkinson, 1991; San Agustin et al., 1998).

4.2.1. Structure of the Regulatory Subunits

The RI and RII subunits contain an amino-terminal dimerization domain, a region responsible for interaction with the C-subunit, and in the carboxy-terminus, two tandem cAMP binding sites, termed A and B (Corbin et al., 1978; Doskeland, 1978). Of the two cAMP binding sites that are located in the C-terminal domain, only site B is exposed in the inactive tetrameric PKA complex (Doskeland et al., 1993). Binding of cAMP to this site enhances binding of cAMP to the site A in a positively co-operative fashion, as a result of a conformational change in the molecule.

4.2.2. Structure of the Catalytic-Subunits

The C-subunits retain the catalytic core motif that is common to all protein kinases (Hanks et al., 1988, Taylor et al., 1992). The C-subunits are bi-lobed globular proteins, with a small amino-terminal and a larger carboxyl-terminal lobe involved in peptide binding and catalysis. Cα and Cβ contain a domain that is capable of binding the specific peptide inhibitor of PKA, PKI (Wen et al., 1994). PKI, which contains a nuclear export signal (NES), appears to have the ability of transporting the C-subunit from the nucleus to the cytosol, and may serve as a major regulator of C-subunit activity in vivo (Wen et al., 1995).

4.2.3. Levels and Expression of the Regulatory and Catalytic-Subunits

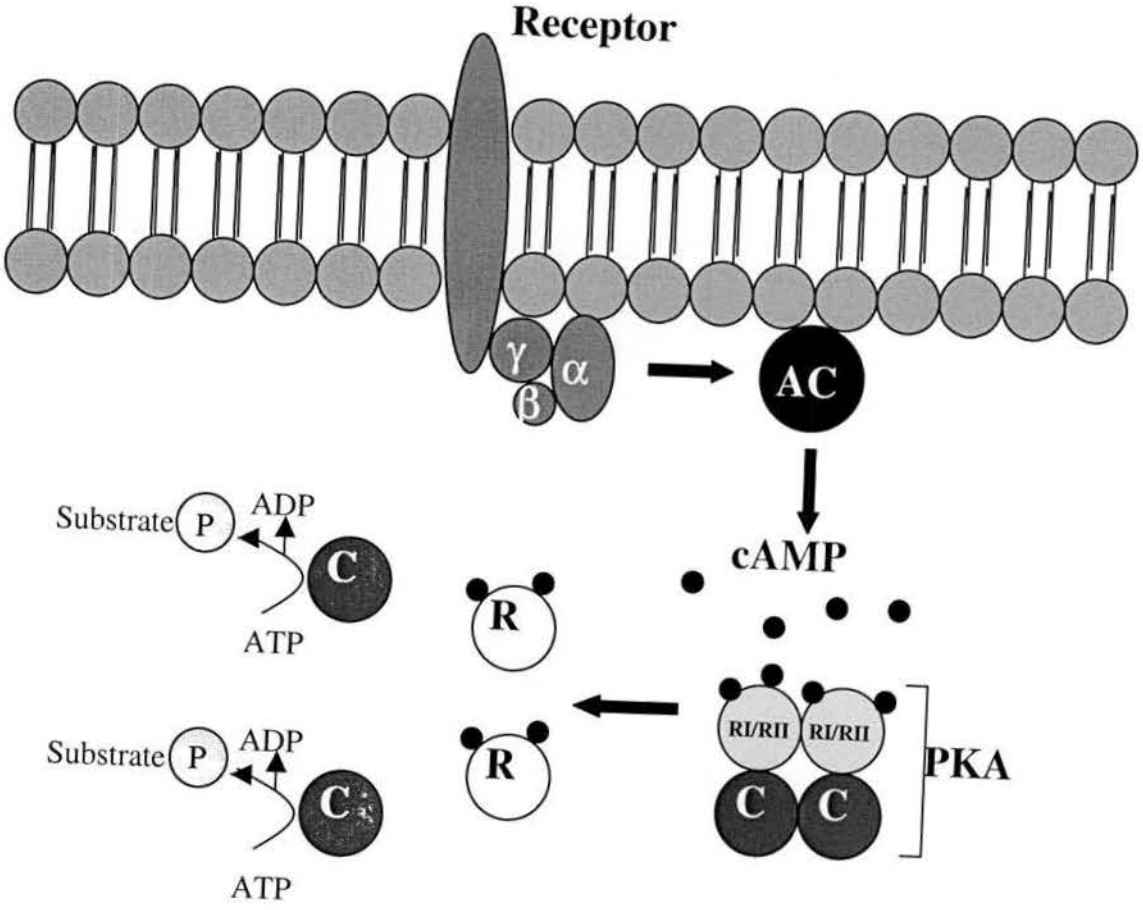
Studies by Cadd and McKnight (1989) revealed that in mice, RIα is expressed in the heart and central nervous system (CNS), whereas RIβ expression is more restricted to nervous tissues such as the spinal cord and the brain. Furthermore, RIIα and RIIβ show distinct patterns of expression, with RIIα predominantly expressed in the heart and RIIβ expressed in the liver and fat tissue, whilst both are expressed in the brain (Cummings et al., 1989). It is generally assumed that the C-subunits associate freely with dimers of all the R-subunits. However, PKA I holoenzymes are more readily dissociated by cAMP in vitro than PKA II holoenzymes (Beebe and Corbin, 1986; Dostmann et al., 1990). Furthermore, studies have shown that the C-subunit will preferentially bind to RII and leave the RI present as free dimers (Otten and McKnight, 1989), which indicates that PKA II holoenzymes are assembled preferentially over PKA I under physiological conditions. In addition to their unique expression patterns across tissues, the various PKA subunits show preferential distribution subcellularly. RII subunits are generally found in the particulate fraction of neural homogenates. This is attributable to their high affinity for a group of anchoring proteins, A-kinase anchoring proteins (AKAPs) which bind PKA (predominantly type II) to various subcellular locations and are now believed to act as scaffolds supporting multi-protein signal transduction complexes that can also include other kinases and phosphatases (Coghlan et al., 1995; Faux and Scott, 1996). Histologically, RII subunits have been found to be associated with structures such as the nuclear envelope, the golgi apparatus, and the dendritic cytoskeleton (Diviani and Scott, 2001; Skalhegg and Tasken, 2000). In contrast RI subunits tend to be found in the cytosolic fraction of cells, although this may be simply due to the low affinity of RI subunits for binding proteins (Skalhegg et al., 1994).

Taken together, this demonstrates the existence of multiple R and C-subunits harbouring different biochemical features and activities. When assembled, they may give rise to a number of PKA holoenzymes with different biological characteristics and activities. A number of different PKA holoenzymes may certainly account for some of the specificity seen in the cAMP / PKA pathway.

Figure 4.1 The cAMP Signal Transduction Cascade

cAMP is generated from ATP when a ligand binds to a G protein coupled receptor that activates adenylate cyclase (AC). PKA is a holoenzyme consisting of regulatory (R) and catalytic (C) subunits. When associated as a holoenzyme the R-subunits restrain the C-subunits in a catalytically inactive state. Activation of PKA occurs when four molecules of cAMP bind to the R-subunit dimer, two to each subunit. When both cAMP binding sites are occupied the R-subunit adopts a conformation with low affinity for the C-subunit and the holoenzyme dissociates.

The cAMP / PKA pathway is known to be activated by a number of different receptors that upon binding of their respective ligands, transduce signals over the cell membrane by coupling G proteins. These G proteins interact with adenylate cyclase on the inner membrane surface either to activate or inhibit the production of cAMP. Receptors that activate PKA, through the generation of cAMP, regulate a vast number of cellular processes, such as metabolism (Krebs and Beavo, 1979), gene regulation (Roesler et al., 1988), cell growth and division (Whitfield et al., 1979), cell differentiation (Liu, 1982; Schwartz and Rubin, 1983) and sperm motility (Tash et al., 1984) as well as ion channel conductivity (Li et al., 1993).


Receptor
γ
α
β
AC
cAMP
Substrate
P
ADP
C
ATP
R
RI/RII
RI/RII
PKA
C
C
Substrate
P
ADP
C
ATP
R

4.3. cAMP Signal Transduction Cascade and Sensory Transmission

The results in chapter 3 have suggested the importance of the $VPAC_2$ receptor in nociceptive processing following peripheral nerve injury. There is considerable evidence suggesting that the underlying mechanisms of persistent pain result from long term changes in nociceptive processing of peripheral sensory neurones (peripheral sensitisation) and / or neurones of the CNS (central sensitisation). While membrane receptors certainly contribute to the initiation of peripheral and central sensitisation, it is likely that second messenger-activated changes in neuronal activity account for the maintenance of persistent pain states.

Pre-synaptic transmitter / neuromodulator release (glutamate / substance P) results in changes in signal transduction pathways in dorsal horn neurones and the activation of ligand-gated ion channels (such as AMPA and NMDA receptors), but also second messenger-linked receptors (such as mGluR and NK-1 receptors). Activation of these multiple receptors results in an increase in intracellular Ca^{2+}, both via Ca^{2+} inflow and release from intracellular stores (Mayer and Miller, 1991). These pathways and / or Ca^{2+} elevation per se can lead to production of a number of second messengers including (IP_3, DAG), and cAMP, which is also generated more directly via other G protein coupled receptors such as the VPAC / PAC receptors. These second messengers and Ca^{2+} lead to activation of key protein kinases such as CaM kinase, PKC and PKA. There is now considerable evidence to suggest that intracellular second messenger activated protein kinases are important for the development of increased neuronal excitability and persistent alterations in nociceptive processing.

After stimulation, the activated protein kinases may phosphorylate various substrate proteins, including ion channels, G protein coupled receptors and other enzymes to enhance neuronal function and pain signalling (Taiwo et al., 1989; Taiwo and Levine, 1991). One of the downstream targets for these kinases are membrane bound receptors / ion-channels, of which the NMDA and AMPA are the best characterised. Phosphorylation of the NMDA receptor leads to post-translational modification of the receptor resulting in dramatic changes in NMDA-receptor channel kinetics and a

reduction in its voltage-dependent Mg^{2+} block. Both of these changes augment subsequent responsiveness to synaptically released glutamate, increasing synaptic strength, membrane excitability and thereby eliciting central sensitisation and thus enabling previously sub-threshold inputs to drive the output of the cell. These changes can establish a persistent pain state in which there are enhanced responses to noxious stimuli (hyperalgesia) and non-noxious stimuli can produce pain (allodynia).

Many neurotransmitters and hormones transduce their signal into a cell by activating G protein coupled receptors that modulate adenylate cyclase activity. These ligands include small molecule neurotransmitters, such as acetylcholine, dopamine, noradrenaline, serotonin and histamine, as well as peptide transmitters such as VIP, SOM and NPY (Brandon et al., 1997). The cAMP transduction cascade is associated with several G protein receptors found in the spinal cord such as the prostaglandin receptors (Hintgen et al., 1995) CGRP (Bushfield et al., 1993) substance P (Satoh et al., 1992) opioid (Aantaa et al., 1995) $VPAC_2$ receptors (Lutz et al., 1993) serotonin (Hen., 1993) adrenergic (Aantaa et al., 1995) and metabotropic glutamate receptors (Schoepp and Johnson, 1993).

Increasing evidence suggests that the cAMP transduction pathway is implicated in the establishment of prolonged changes in the excitability of primary afferent nociceptors and dorsal horn neurones (Taiwo et al., 1989; Taiwo and Levine, 1991; Cerne et al., 1992, 1993; Mao et al., 1993; Palecek et al., 1994; Lin et al., 1996; Sluka, 1997; Malmberg et al., 1997). The sensitisation of primary afferent neurones that occurs in the setting of inflammation has been shown to involve cAMP and PKA-dependent mechanisms (England et al., 1996; Kress et al., 1996). Peripherally cAMP acts in nociceptive primary afferents as a second messenger to mediate the decrease in mechanical nociceptive threshold produced by a number of inflammatory mediators that are thought to act at different receptors on the peripheral terminals of primary afferents. In fact all steps of the cAMP second messenger cascade have been shown to be necessary for the induction of hyperalgesia by direct acting agents (Taiwo et al., 1989). Forskolin, which directly activates adenylate cyclase, results in a lowering of nociceptive thresholds, and the duration of this hyperalgesia is

prolonged by phosphodiesterase inhibitors (Ferreira and Nakamura, 1979). The lowering of pain threshold produced by paw injection of prostaglandin E_2 (PGE_2) can be reduced by inhibiting PKA (Taiwo et al., 1989; Taiwo and Levine, 1991). Also, the increases in the conductance of TTX-resistant Na^+ channel by PGE_2 (predominantly expressed on small diameter nociceptive neurones), (Akopian et al., 1996) are mimicked by cAMP (England et al., 1996; Gold et al., 1996). Activators of PKA, including cAMP analogues, have been shown to enhance peptide release from cultured sensory neurones (Hintgen et al., 1995; Supowit et al., 1995). Furthermore cAMP-dependent phosphorylation of Ca^{2+} channels has also been demonstrated, suggesting that PKA is one essential component of the mechanisms of enhanced neurotransmitter release during persistent nociception (Hell et al., 1995). The peripheral component of hyperalgesia is independently maintained by PKA and inhibitors of PKA can inhibit the catalytic-subunit-induced hyperalgesia at any point during its time course. This suggests that hyperalgesia is not maintained by very persistent changes in the cell downstream from PKA, such as long lasting enhancement of ion-channels (Aley and Levine, 1999).

Centrally, the role of cAMP in spinal nociceptive processing is less clear. Injection of the cAMP analogue 8-Br-cAMP or the catalytic-subunits of PKA enhances the responses of dorsal horn neurones to glutamate gated ion channel activation (Cerne et al., 1992; 1993). Activation of the cAMP transduction cascade at the spinal cord level results in mechanical hyperalgesia and allodynia and it has been demonstrated that the secondary mechanical allodynia following intradermal injection of capsaicin is mediated by this transduction cascade (Sluka et al., 1997). Also, blockade of PKA with H89 by microdialysis infusion in the dorsal horn reduces the sensitisation of STT cells to capsaicin injection (Sluka et al., 1997b), and capsaicin-induced mechanical hyperalgesia in rats (Sluka and Willis, 1997). While selective inhibitors of the PKA isoforms are not available, mutant mice that lack one of the subunits have been developed to study the function of PKA isoforms. In mice that selectively lack the RIβ subunit (Brandon et al., 1995) the induction of the transcription factor C-fos in the spinal dorsal horn by intraplantar formalin injection is significantly reduced providing a molecular indicator of the loss of function in these mice

(Malmberg et al., 1997). It is believed that RIβ is a critical pre-synaptic PKA subunit, in this case for nociceptive processing in the terminals of primary afferent fibres (Malmberg et al., 1997). Thus, it appears that the cAMP transduction cascade at a central site is involved in maintaining secondary hyperalgesia and allodynia (Sluka et al., 1997), and activation of the cAMP pathway must occur in response to mechanisms responsible for inducing central sensitisation (Sluka, 1997). These studies are consistent with other studies suggesting that PKA plays a role in prolonged changes in neuronal synaptic efficacy, which include studies of long term potentiation in the hippocampus (Frey et al., 1993; Huang et al., 1995).

4.4. Aims of the Present Experiments

This study aimed to investigate the possible role of PKA in the abnormal behavioural responses following CCI, and to identify any specific alterations in the expression of the subunits of PKA following CCI. To assess the contribution of PKA in the maintenance of thermal hyperalgesia, and mechanical and cold allodynia following CCI, intrathecal injection of a number of inhibitors of PKA (H89, myr-PKI (5-24) and Rp-8-CPT-cAMPs) was carried out in neuropathic rats and normal controls. Since several isoforms of PKA exist, its involvement in nociception may be related to one specific isoform, and / or alteration in the expression of various isoforms. To assess any alterations in the levels of expression of PKA subunits in the lumbar dorsal horn following CCI, in situ hybridisation histochemistry and Western blot analysis of the expression of mRNA and protein for the catalytic (Cα, Cβ) and regulatory (RIα, RIβ, RIIα and RIIβ) subunits of PKA was carried out.

4.5. Results

4.5.1. Effects of Intrathecal Injection of the Highly Selective PKA Inhibitors H89, myr-PKI (5-24) and Rp-8-CPT-cAMPs (Rp-cAMPs) on Reflex Withdrawal Responses in Normal and CCI Rats

These results were obtained from a total of 117 rats (n=99 CCI and n=18 normal). Intrathecal injection of the selective PKA inhibitors H89, myr-PKI (5-24) and Rp-8-CPT-cAMPs was carried out as described in previously (Chapter 2 section 2.3.4). Behavioural measurements commenced at 15 min post-injection and continued until full recovery was observed or for a minimum testing period of 90 min. In order to differentiate between the contribution of PKA catalytic-subunit activity compared to activation of PKA by cAMP, several different inhibitors of PKA were administered. H89 and myr-PKI (5-24) inhibit the catalytic activity of PKA by inhibiting the catalytically active C-subunits. Rp-8-CPT-cAMPS however, is a competitive inhibitor of cAMP and sequesters PKA as an inactive holoenzyme. All three inhibitors, H89, myr-PKI (5-24) and Rp-8-CPT-cAMPs significantly reversed the ipsilaterally enhanced sensory reflex withdrawal responses to noxious heat measures, innocuous mechanical stimulation and innocuous cold, believed to be indicative of thermal hyperalgesia, mechanical and cold allodynia respectively. No significant effects were observed on the reflex responses elicited from the contralateral limb (Figures 4.2 – 4.12).

Injection of increasing concentrations of H89 resulted in a characteristic dose dependent inhibition (Figures 4.2, 4.3 and 4.4). Injection of Myr-PKI (5-22) and Rp-8-CPT-cAMPs (Figure 4.6, 4.7, 4.8, 4.9 and 4.10) had a similar effect. Intrathecal injection of vehicle had no effect on baseline values in all three sensory tests (Figure 5.5, 5.6, 5.7) and injection of the inhibitors of PKA in normal animals also had no effect on baseline values (Figure 4.5). An additional control involved the injection of a myristoylated inactive control peptide in CCI rats which had no significant effect on reflex withdrawal responses (Figure 4.7). The effects of all three inhibitors appeared to be specific to the ipsilateral limb following CCI with no apparent effect on either the contralateral limb or baseline values in normal unoperated rats.

Figure 4.2 Effects of Intrathecal Injection of the PKA Inhibitor H89 on Reflex Withdrawal Responses to Noxious Heat in CCI Rats

Data are presented as mean paw withdrawal latency for ipsilateral and contralateral paws plotted against time (min) pre- and post-injection. Arrow marks intrathecal drug injection. In rats exhibiting peak behavioural changes following CCI, paw withdrawal latency to noxious heat ipsilateral (but not contralateral) to nerve injury showed significant differences between pre- and post-drug injection values († $p \leq 0.05$; one way ANOVA followed by Neuman-Keuls post-hoc test). Significant differences between contralateral and ipsilateral paw withdrawal latency are indicated (* $p \leq 0.05$; Student's paired t-test). A dose dependent effect was observed following injection of H89 (at arrow) in that the recovery was faster at lower doses. (A) Effects of intrathecal injection of H89 ([2nmol in 50µl], n=9). (B) Effects of intrathecal injection of H89 ([6nmol in 50µl], n=9). (C) Effects of intrathecal injection of H89 ([20nmol in 50µl], n=9).

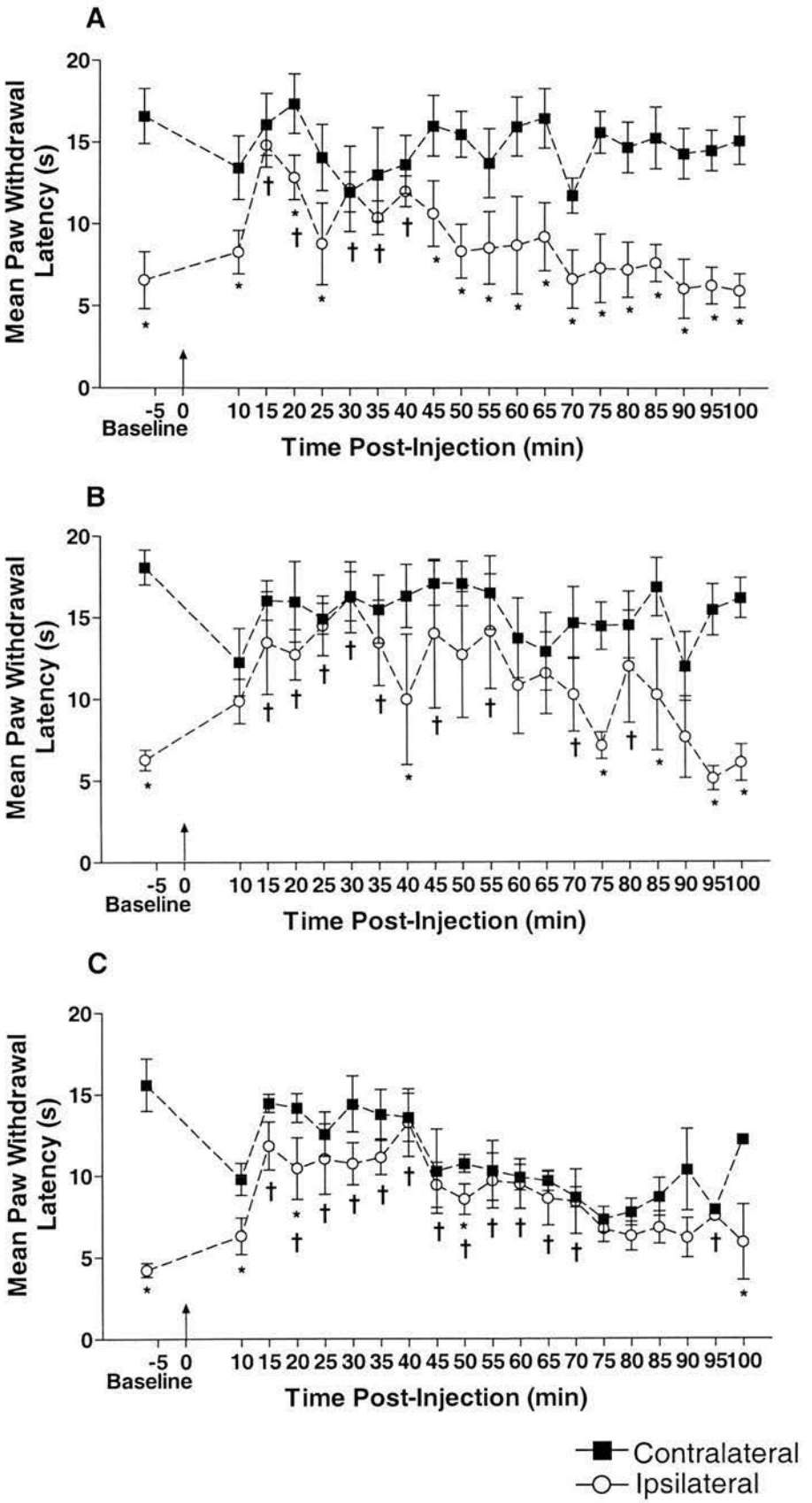

A
B
C
Mean Paw Withdrawal Latency (s)
0
5
10
15
20
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95100
Time Post-Injection (min)
Contralateral
Ipsilateral

Figure 4.3 Effects of Intrathecal Injection of the PKA Inhibitor H89 on Reflex Withdrawal Responses to Innocuous Mechanical Stimuli in CCI Rats

Data are presented as mean paw withdrawal threshold to innocuous mechanical stimulation for ipsilateral and contralateral paws plotted against time (min) pre- and post-injection. Arrow marks intrathecal drug injection. In rats exhibiting peak behavioural changes following CCI, paw withdrawal threshold to innocuous mechanical stimuli ipsilateral (but not contralateral) to nerve injury showed significant differences between pre- and post-drug injection values († $p \leq 0.05$; Kruskall-Wallis ANOVA followed by a Dunn's post-hoc test). Significant differences between contralateral and ipsilateral paw withdrawal threshold are indicated (* $p \leq 0.05$; Mann-Whitney U test). A dose dependent effect was observed following injection of H89. (A) Effects of intrathecal injection of H89 ([2nmol in 50µl], n=9). (B) Effects of intrathecal injection of H89 ([6nmol in 50µl], n=9). (C) Effects of intrathecal injection of H89 ([20nmol in 50µl], n=9).

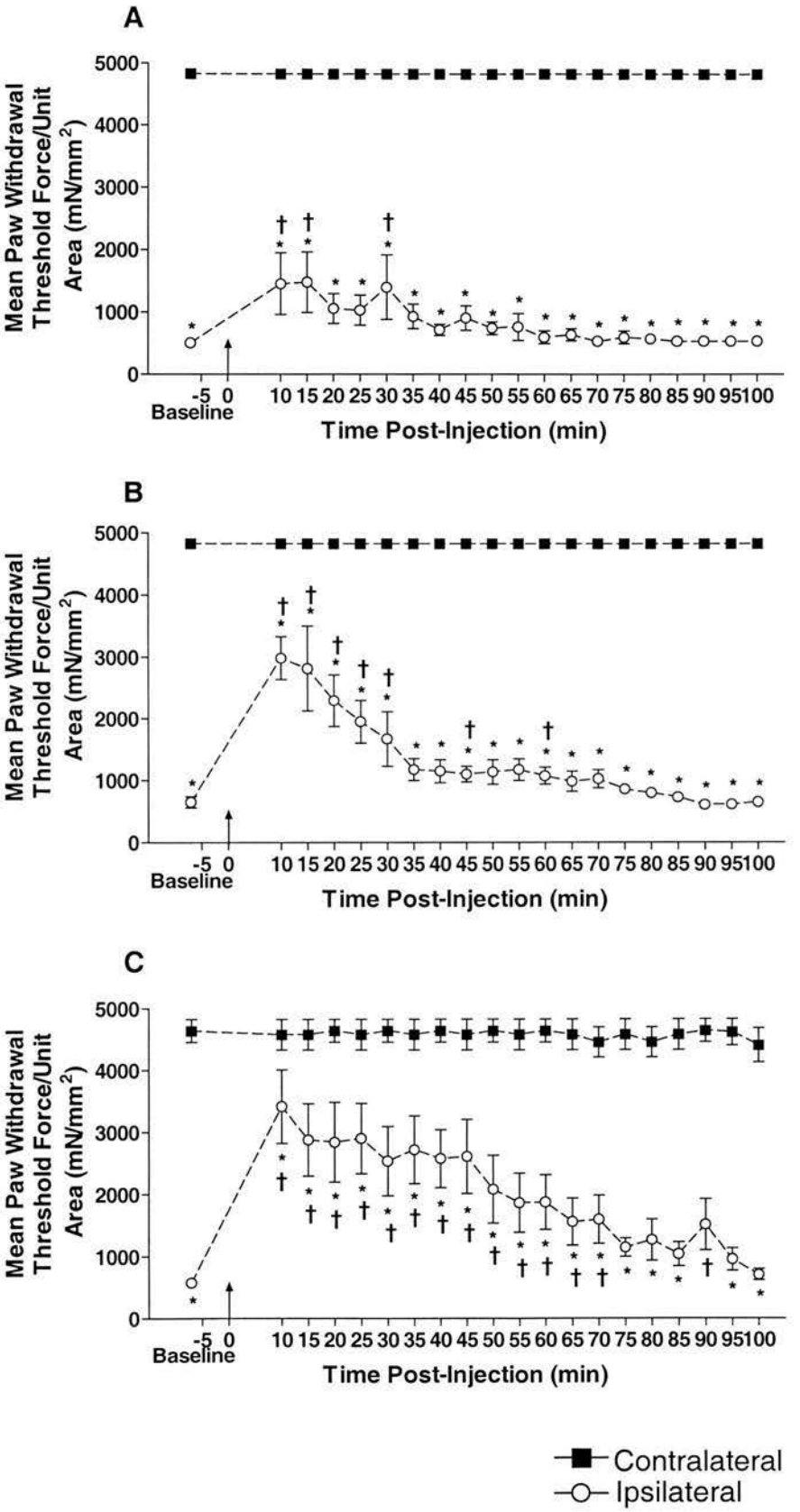

A
B
C
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm^2)
5000
4000
3000
2000
1000
0
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95100
Time Post-Injection (min)
Contralateral
Ipsilateral

Figure 4.4 Effects of Intrathecal Injection of the PKA Inhibitor H89 on Reflex Withdrawal Responses to Innocuous Cold Stimuli in CCI Rats

Data are presented as mean suspended paw elevation time (SPET) (s) for ipsilateral and contralateral paws plotted against time (min) pre- and post-injection. Arrow marks intrathecal drug injection. In rats exhibiting peak behavioural changes following CCI, suspended paw elevation time (SPET) ipsilateral (but not contralateral) to nerve injury showed significant differences between pre- and post-drug injection values († $p \leq 0.05$; one way ANOVA followed by Neuman-Keuls post-hoc test). Significant differences between contralateral and ipsilateral paw withdrawal duration are indicated (* $p \leq 0.05$; Student's paired t-test). (A) Effects of intrathecal injection of H89 ([6nmol in 50μl], n=9).

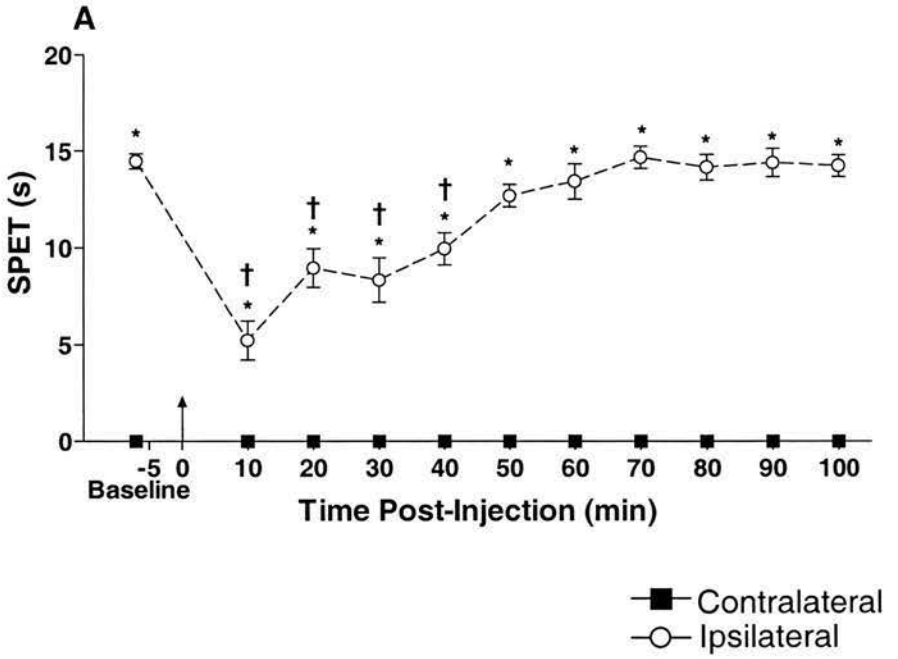

A
SPET (s)
20
15
10
5
0
-5
Baseline
0
10
20
30
40
50
60
70
80
90
100
Time Post-Injection (min)
Contralateral
Ipsilateral

Figure 4.5 Effects of Intrathecal Injection of the PKA Inhibitor H89 on Reflex Withdrawal Responses to Noxious Heat and Innocuous Mechanical Stimuli in Normal Rats

Data are presented as mean paw withdrawal latency (s) from noxious heat (A), and mean paw withdrawal threshold (mN / mm^2) to innocuous mechanical stimuli (B) for left and right paws plotted against time (min) pre- and post-injection. Arrow marks intrathecal drug injection.

In normal unoperated rats, paw withdrawal latency to noxious heat (A) and paw withdrawal threshold to innocuous mechanical stimulation (B) was unaltered following intrathecal injection of H89 (Neuman-Keuls one way ANOVA and Kruskall-Wallis ANOVA respectively). H89 was injected at a dose of [6nmol in 50μl], n=6.

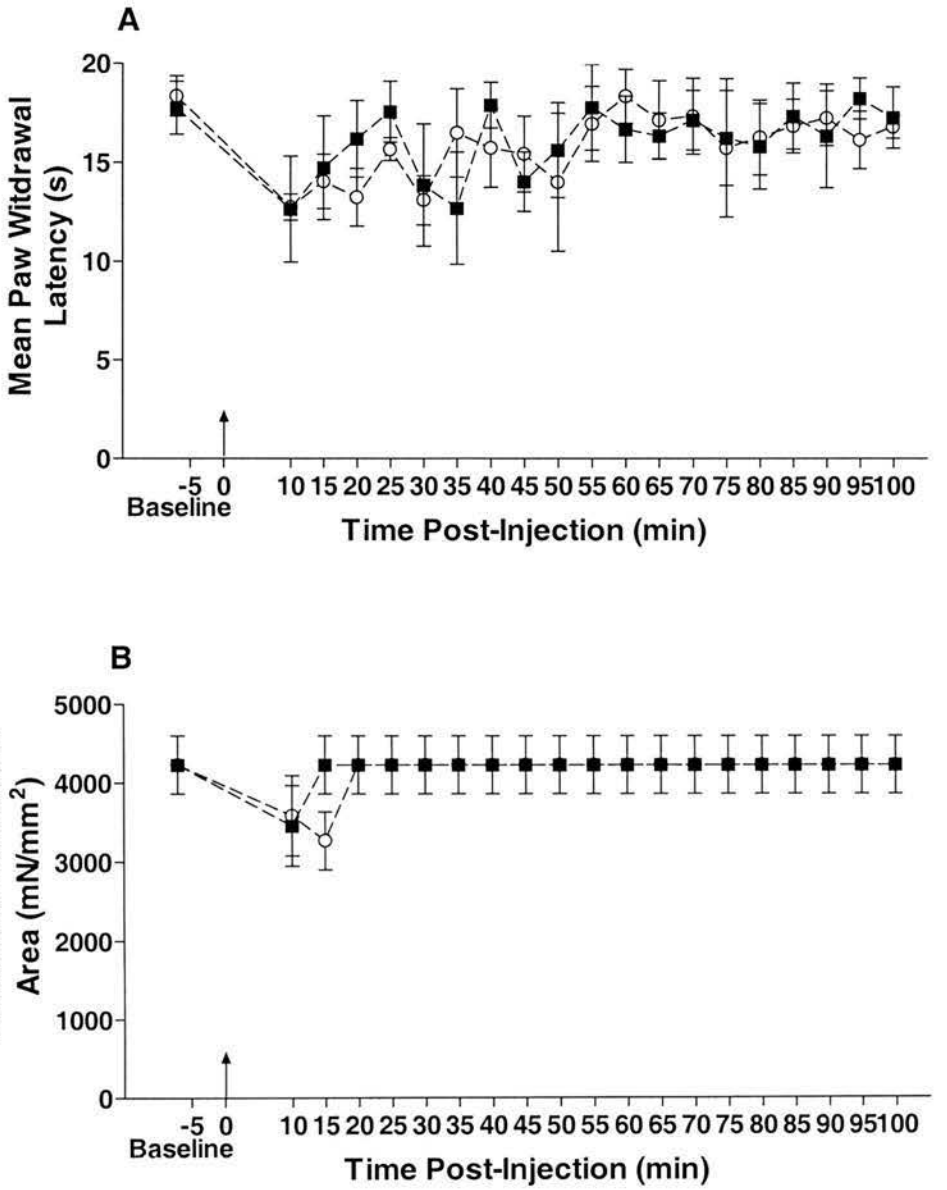

A
Mean Paw Witdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95100
Time Post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm2)
5000
4000
3000
2000
1000
0
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95100
Time Post-Injection (min)

Figure 4.6 Effects of Intrathecal Injection of the PKA Inhibitor myr-PKI (5-24) on Reflex Withdrawal Responses to Noxious Heat, Innocuous Mechanical and Innocuous Cold Stimuli in CCI Rats

Data are presented as mean paw withdrawal latency (s) from noxious heat (A), mean paw withdrawal threshold (mN / mm^2) to normally innocuous mechanical stimuli (B) and mean suspended paw elevation time (s) from normally innocuous cold (C) for ipsilateral and contralateral paws plotted against time (min) pre- and post-injection.

(A) In rats exhibiting peak behavioural changes following CCI, paw withdrawal latency to noxious heat ipsilateral (but not contralateral) to nerve injury showed significant differences between pre- and post-drug injection values († $p \leq 0.05$; one way ANOVA followed by Neuman-Keul's post-hoc test). Significant differences between contralateral and ipsilateral paw withdrawal latency are indicated (* $p \leq 0.05$; Student's paired t-test). myr-PKI (5-24)was injected at a dose of [2nmol in 50µl], (n=9).

(B) In rats exhibiting peak behavioural changes following CCI, paw withdrawal threshold to innocuous mechanical stimulation ipsilateral (but not contralateral) to nerve injury showed significant differences between pre- and post-drug injection values († $p \leq 0.05$; Kruskal-Wallis ANOVA followed by a Dunn's post-hoc test). Significant differences between contralateral and ipsilateral paw withdrawal threshold are indicated (* $p \leq 0.05$; Mann-Whitney U test). myr-PKI (5-24) was injected at a dose of [2nmol in 50µl], (n=9).

(C) In rats exhibiting peak behavioural changes following CCI, suspended paw elevation time (SPET) ipsilateral (but not contralateral) to nerve injury showed significant differences between pre- and post-drug injection values († $p \leq 0.05$; one way ANOVA followed by Neuman-Keuls post-hoc test). Significant differences between contralateral and ipsilateral paw withdrawal duration are indicated (* $p \leq 0.05$; Student's paired t-test). myr-PKI (5-24) was injected at a dose of [2nmol in 50µl], n=9.

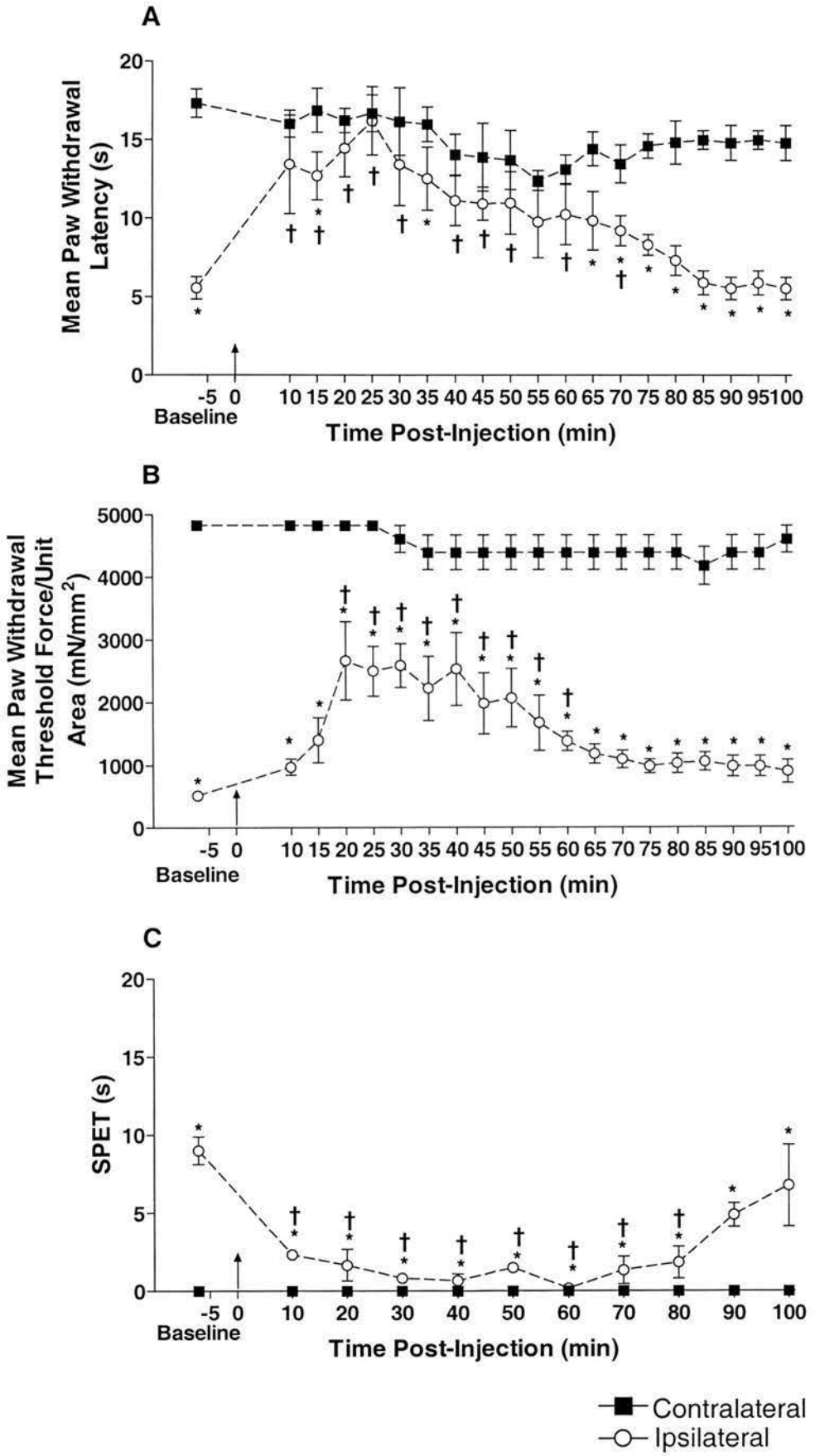

A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95100
Time Post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm2)
5000
4000
3000
2000
1000
0
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95100
Time Post-Injection (min)
C
SPET (s)
20
15
10
5
0
-5 0
Baseline
10 20 30 40 50 60 70 80 90 100
Time Post-Injection (min)
Contralateral
Ipsilateral

Figure 4.7 Effects of Intrathecal Injection of the Myristoylated Control Peptide on Reflex Withdrawal Responses to Noxious Heat, Innocuous Mechanical and Innocuous Cold Stimuli in CCI Rats

Data are represented as mean paw withdrawal latency (s) to noxious heat (A) and mean paw withdrawal threshold (mN / mm^2) to innocuous mechanical stimuli (B), for ipsilateral and contralateral paws plotted against time (min) pre- and post-injection. Arrow marks intrathecal drug injection.

(A) In rats exhibiting peak behavioural changes following CCI, paw withdrawal latency to noxious heat ipsilateral and contralateral to nerve injury was unchanged compared to pre- and post-drug injection values (one way ANOVA). Significant differences between contralateral and ipsilateral paw withdrawal latency are indicated (* $p \leq 0.05$ Student's paired t-test). myr-control peptide was injected at a dose of [2nmol in 50µl], (n=6).

(B) In rats exhibiting peak behavioural changes following CCI, paw withdrawal threshold to mechanical stimulation ipsilateral and contralateral to nerve injury was unchanged compared to pre- and post-drug injection values (Kruskal-Wallis ANOVA). Significant differences between contralateral and ipsilateral paw withdrawal threshold are indicated (* $p \leq 0.05$ Mann-Whitney U test). myr-control peptide was injected at a dose of [2nmol in 50µl], (n=6).

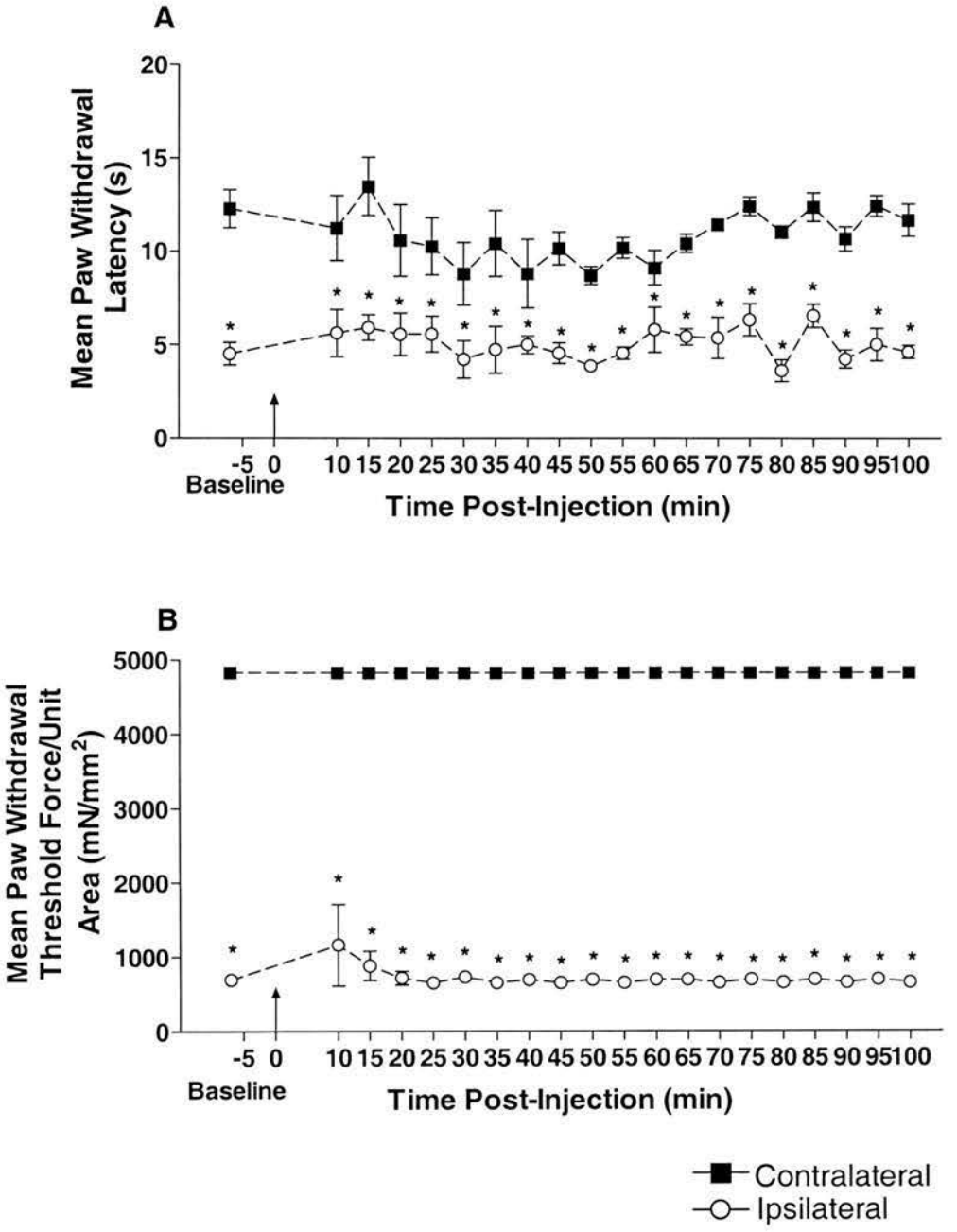

A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Time Post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm2)
5000
4000
3000
2000
1000
0
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Time Post-Injection (min)
Contralateral
Ipsilateral

Figure 4.8 Effects of Intrathecal Injection of the PKA Inhibitor myr-PKI (5-24) on Reflex Withdrawal Response to Noxious Heat and Innocuous Mechanical Stimuli in Normal Rats

Data are presented as mean paw withdrawal latency (s) from noxious heat (A), mean paw withdrawal threshold (mN / mm^2) to innocuous mechanical stimuli (B) for left and right paws plotted against time (min) pre- and post-injection. Arrow marks intrathecal drug injection.

(A) In normal unoperated rats, paw withdrawal latency to noxious heat was unchanged compared to pre- and post-drug injection values (one way ANOVA). There was no significant difference between left and right paw withdrawal latency (Student's paired t-test). Myr-PKI (5-24) was injected at a dose of [2nmol in 50µl], (n=6).

(B) In normal unoperated rats, paw withdrawal threshold to innocuous mechanical stimuli was unchanged compared to pre- and post-drug injection values (Kruskal-Wallis ANOVA). There was no significant difference between left and right paw withdrawal threshold (Mann-Whitney U test). Myr-PKI (5-24) was injected at a dose of [2nmol in 50µl], (n=6).

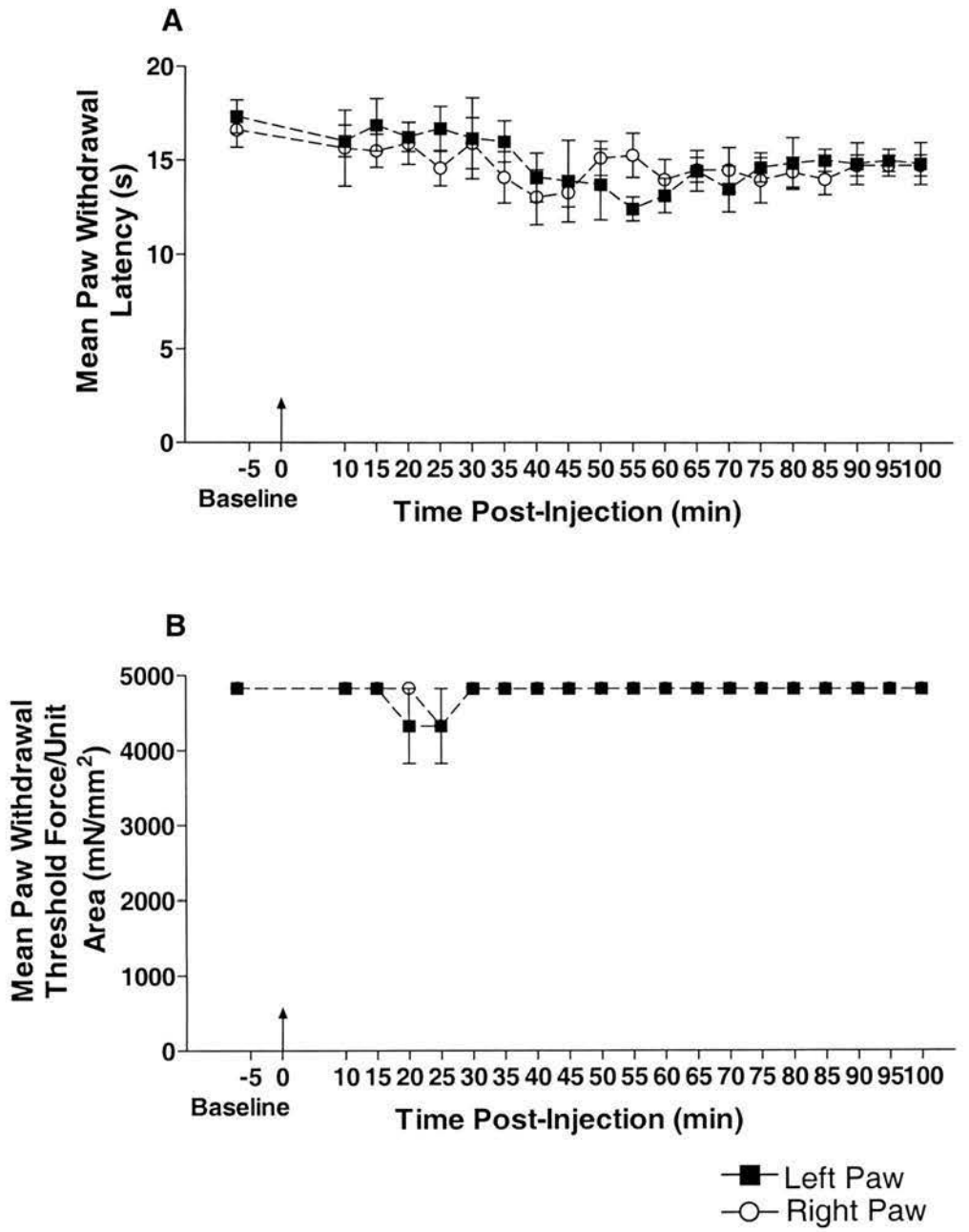

A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95100
Time Post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm2)
5000
4000
3000
2000
1000
0
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95100
Time Post-Injection (min)
Left Paw
Right Paw

Figure 4.9 Effects of Intrathecal Injection of the PKA Inhibitor Rp-8-CPT-cAMPs on Reflex Withdrawal Responses to Noxious Heat in CCI Rats

Data are presented as mean paw withdrawal latency for ipsilateral and contralateral paws plotted against time (min) pre- and post-injection. Arrow marks intrathecal drug injection. In rats exhibiting peak behavioural changes following CCI, paw withdrawal latency to noxious heat ipsilateral (but not contralateral) to nerve injury was showed significant differences between pre- and post-drug injection values († $p \leq 0.05$; one way ANOVA followed by Neuman-Keuls post-hoc test). Significant differences between contralateral and ipsilateral paw withdrawal latency are indicated (* $p \leq 0.05$; Student's paired t-test). A dose dependent effect (in that recovery was more rapid at lower doses) was observed following injection of Rp-8-CPT-cAMPs. (A) Effects of intrathecal injection of Rp-8-CPT-cAMPs [10nmol in 50µl], (n=9). (B) Effects of intrathecal injection of Rp-8-CPT-cAMPs [30nmol in 50µl], (n=9). (C) Effects of intrathecal injection of Rp-8-CPT-cAMPs [100nmol in 50µl], (n=9).

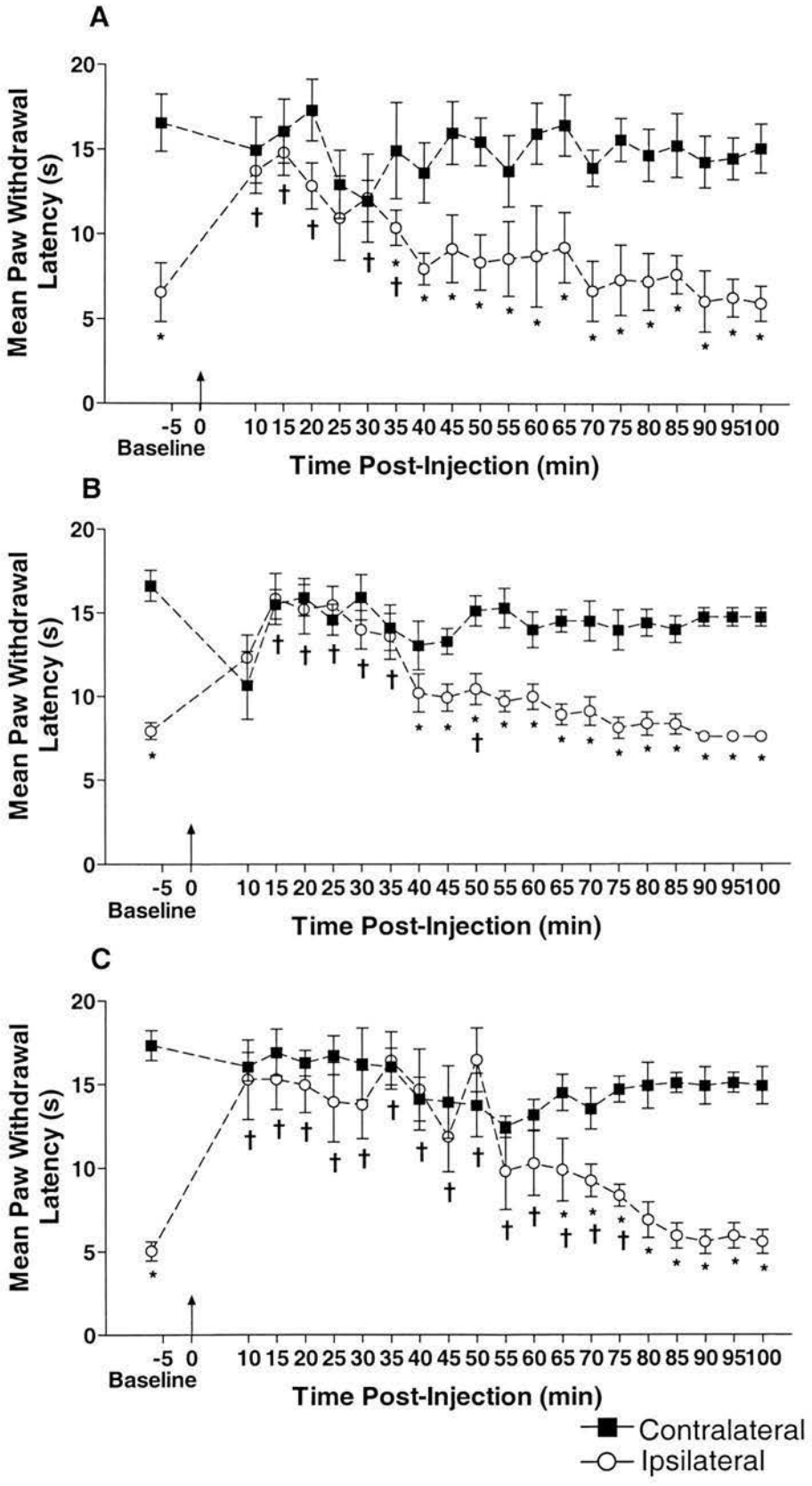

A
B
C
Mean Paw Withdrawal Latency (s)
0
5
10
15
20
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Time Post-Injection (min)
Contralateral
Ipsilateral

Figure 4.10 Effects of Intrathecal Injection of Rp-8-CPT-cAMPS on Reflex Withdrawal Responses to Innocuous Mechanical Stimuli in CCI Rats

Data are presented as mean paw withdrawal threshold to innocuous mechanical stimulation for ipsilateral and contralateral paws plotted against time (min) pre- and post-injection. Arrow marks intrathecal drug injection. In rats exhibiting peak behavioural changes following CCI, paw withdrawal threshold to innocuous mechanical stimuli ipsilateral (but not contralateral) to nerve injury showed significant differences between pre- and post-drug injection values († $p \leq 0.05$; Kruskall-Wallis ANOVA followed by a Dunn's post-hoc test). Significant differences between contralateral and ipsilateral paw withdrawal threshold are indicated (* $p \leq 0.05$; Mann-Whitney U test). Selective ipsilateral effects were observed following injection of Rp-8-CPT-cAMPs. (A) Effects of intrathecal injection of Rp-8-CPT-cAMPs [10nmol in 50µl], (n=9). (B) Effects of intrathecal injection of Rp-8-CPT-cAMPs [30nmol in 50µl], (n=9). (C) Effects of intrathecal injection of Rp-8-CPT-cAMPs [100nmol in 50µl], (n=9).

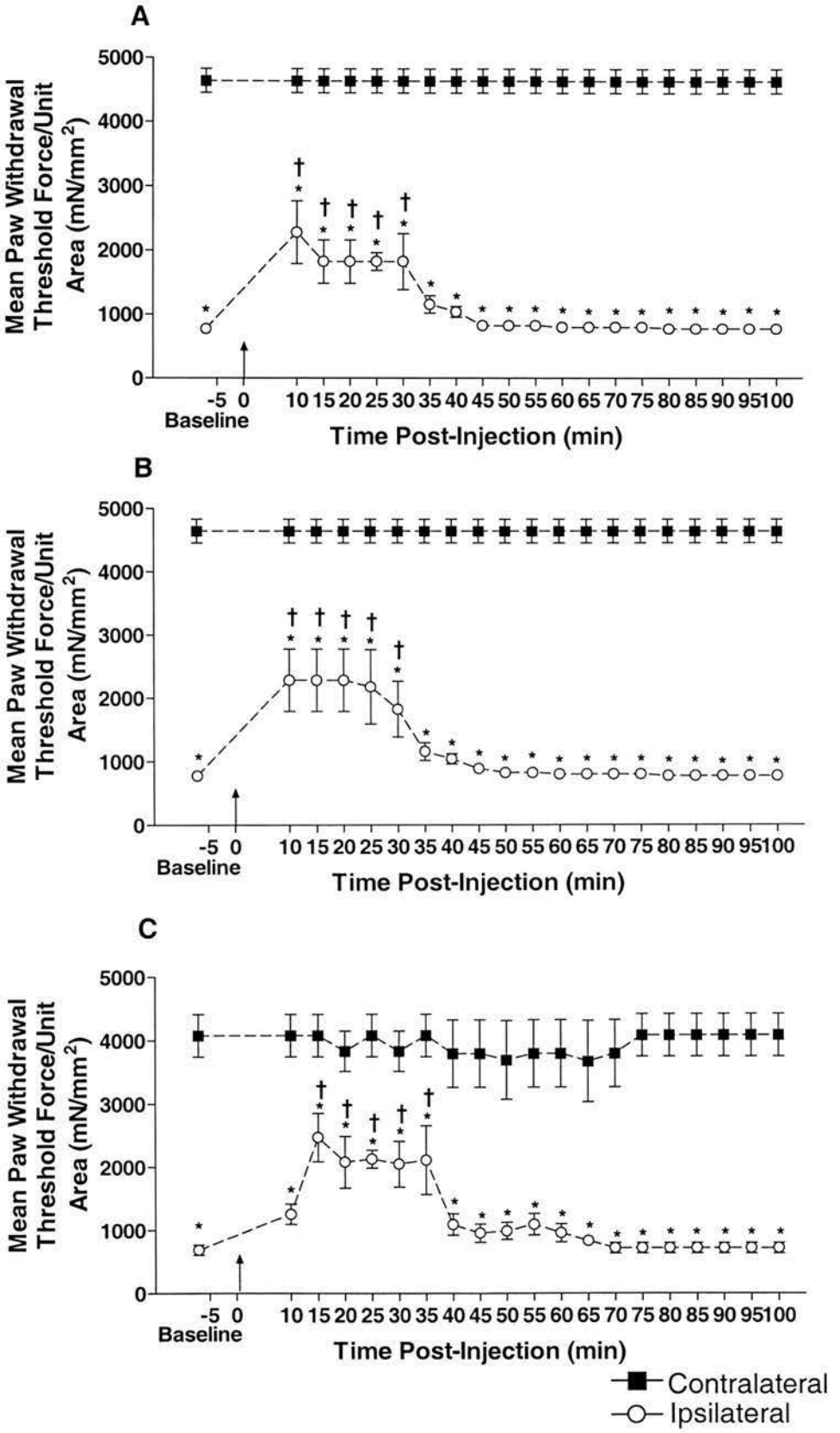

A
B
C
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm²)
5000
4000
3000
2000
1000
0
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Time Post-Injection (min)
Contralateral
Ipsilateral

Figure 4.11 Effects of Intrathecal Injection of Rp-8-CPT-cAMPs on Reflex Withdrawal Responses to Innocuous Cold Stimuli in CCI Rats

Data are presented as mean suspended paw elevation time (SPET) (s) for ipsilateral and contralateral paws plotted against time (min) pre- and post-injection. Arrow marks intrathecal drug injection. In rats exhibiting peak behavioural changes following CCI, suspended paw elevation time (SPET) ipsilateral (but not contralateral) to nerve injury showed significant differences between pre- and post-drug injection values († $p \leq 0.05$; one way ANOVA followed by Neuman-Keuls post-hoc test). Significant differences between contralateral and ipsilateral paw withdrawal duration are indicated (* $p \leq 0.05$; Student's paired t-test). (A) Effects of intrathecal injection of Rp-8-CPT-cAMPs [30nmol in 50µl], (n=9).

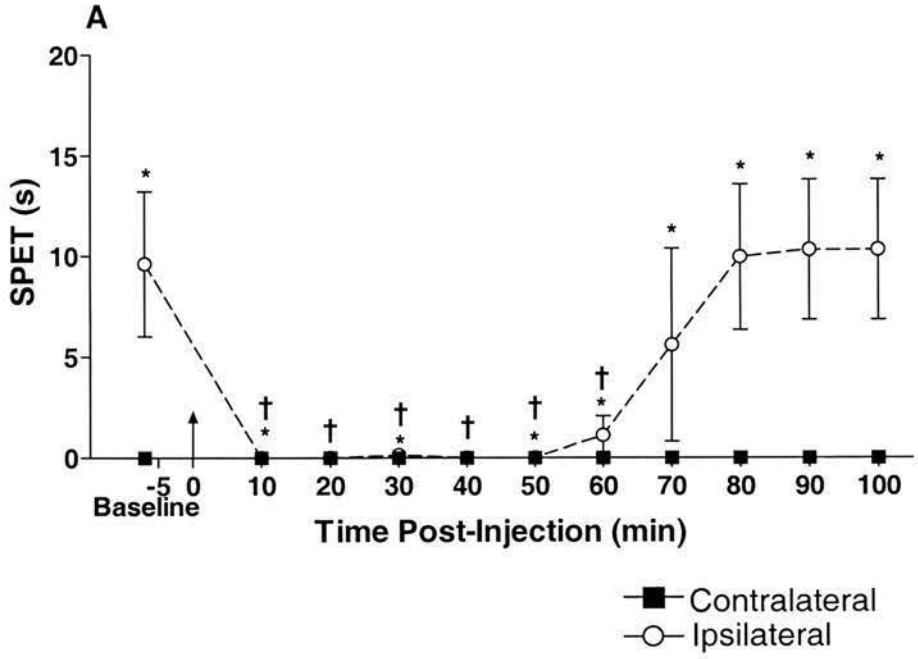
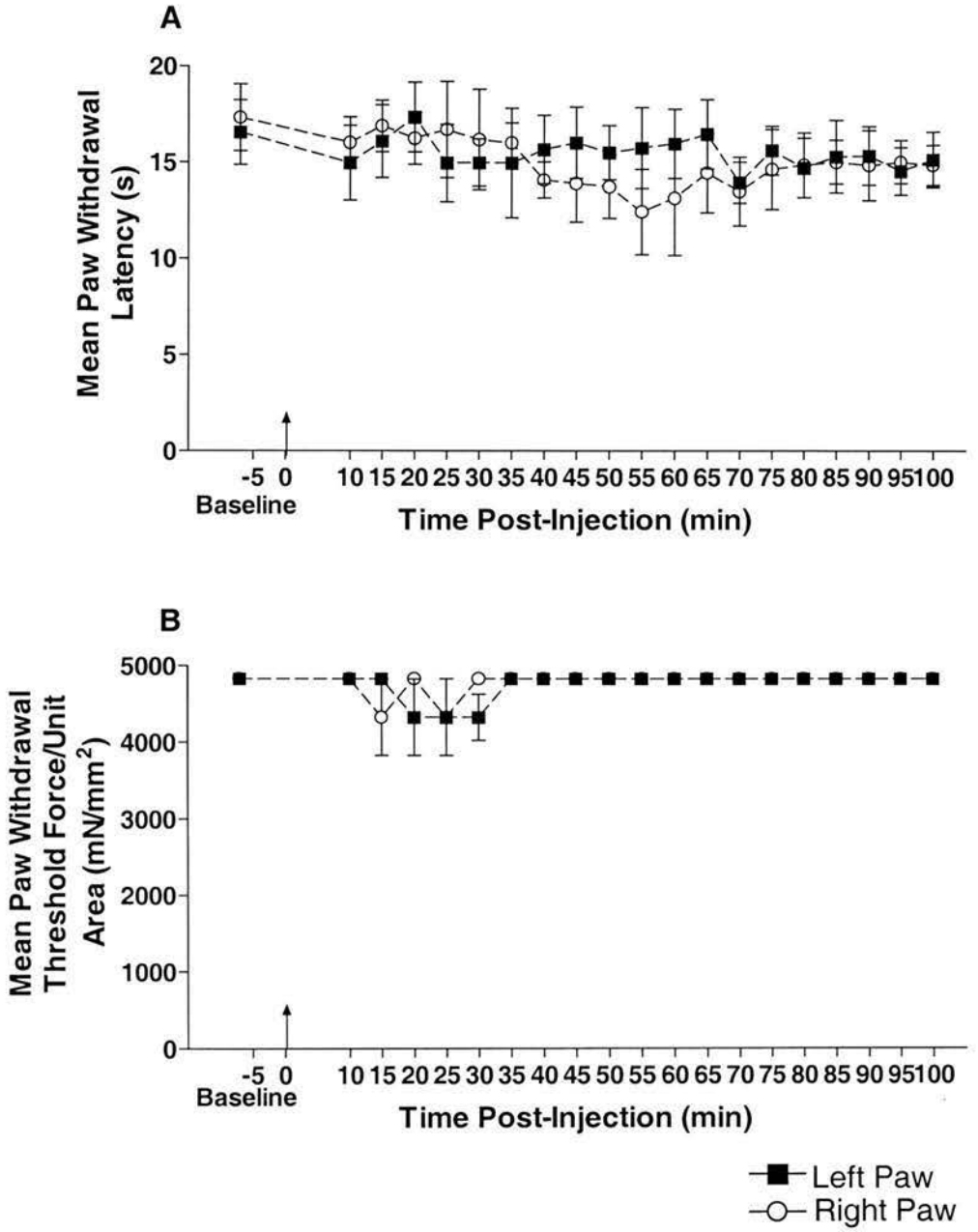

A
SPET (s)
20
15
10
5
0
-5 0
Baseline
10
20
30
40
50
60
70
80
90
100
Time Post-Injection (min)
Contralateral
Ipsilateral

Figure 4.12 Effects of Intrathecal Injection of the PKA Inhibitor Rp-8-CPT-cAMPs on Reflex Withdrawal Responses to Noxious Heat and Innocuous Mechanical Stimuli in Normal Rats

Data are represented as mean paw withdrawal latency (s) from noxious heat (A), mean paw withdrawal threshold (mN / mm^2) to innocuous mechanical stimuli (B) for left and right paws plotted against time (min) pre- and post-injection. Arrow marks intrathecal drug injection.

(A) In normal unoperated rats, paw withdrawal latency to noxious heat was unchanged compared to pre- and post-drug injection values (one way ANOVA). There was no significant difference between left and right paw withdrawal latency (Student's paired t-test). Rp-8-CPT-cAMPs was administered at a dose of [6nmol in 50μl], (n=6).

(B) In normal unoperated rats, paw withdrawal threshold to innocuous mechanical stimuli was unchanged compared to pre- and post-drug injection values (Kruskal-Wallis ANOVA). There was no significant difference between left and right paw withdrawal threshold (Mann-Whitney U test). Rp-8-CPT-cAMPs was administered at a dose of [6nmol in 50μl], (n=6).


A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Time Post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm2)
5000
4000
3000
2000
1000
0
-5 0
Baseline
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Time Post-Injection (min)
Left Paw
Right Paw

4.5.2 Distribution of mRNA for the Catalytic and Regulatory Subunits of cAMP Dependent Protein Kinase (PKA) within the Spinal Dorsal Horn of CCI Rats

The mRNAs for all subunits were distributed widely in spinal cord of normal, sham-operated and CCI rats. The levels of PKA Cβ mRNA were particularly abundant.

4.5.2.1 Catalytic subunits of PKA (Cα and Cβ)

Following CCI of the rat sciatic nerve, there were marked changes in the expression of mRNA for both catalytic-subunits in the dorsal horn ipsilateral to nerve injury, with a particularly large change in the relative levels of Cβ when compared to contralateral values (Figure 4.13). The total number of neurones positively expressing mRNA for Cα and Cβ was significantly increased in the ipsilateral dorsal horn both mediolaterally and laterally in laminae I, II and III (Tables 4.1 and 4.2). Silver grain density was also significantly increased mediolaterally and laterally in LI, II and III (Figures 4.14 and 4.16. There was no significant difference in either cell counts or silver grain densities between contralateral and control values. Within the ventral horn of normal animals, labelling of motoneurones was especially intense (Figure 4.15) but showed no significant change following CCI treatment.

4.5.2.2 Regulatory subunits of PKA (RIα, RIβ, RIIα, and RIIβ)

Following CCI, there were no changes in the relative expression of mRNA for the regulatory subunits of PKA in the spinal dorsal horn following CCI (Figures 4.17, 4.19, 4.21 and 4.23). Both the total number of neurones expressing mRNA for the regulatory subunits and the mean silver grain density were unaltered in all laminae investigated (Tables 4.3, 4.4, 4.5 and 4.6; Figures 4.18, 4.20, 4.22 and 4.24). Control assessment of R subunit mRNA expression in motoneurones appeared unaltered following CCI (data not shown).

Figure 4.13 PKA Cα Subunit mRNA Expression in Lamina I, III and Motoneurones of the Rat Lumbar Spinal Dorsal Horn in CCI (Ipsilateral and Contralateral), Normal and Sham-Operated Rats

Highpower lightfield, black and white photomicrographs showing typical levels of PKA Cα mRNA expression in the mediolateral area of lamina I and III and motoneurones of rat lumbar spinal dorsal horn (scale bars 10μm). Photomicrographs show typical examples of the expression ipsilateral and contralateral to nerve injury and in normal and sham-operated control rats respectively. Positively labelled neurones were identified by a dense accumulation of silver grains (approximately > 5 times background expression) over and around haematoxylin stained nuclei.

Analysis of quantitative densitometry data demonstrated a significant increase in the expression of PKA Cα subunit mRNA ipsilateral to nerve injury when compared to contralateral and control tissue in the superficial laminae of the dorsal horn (A). However, in motoneurones (B) there was no significant alteration in the expression of PKA Cα mRNA ipsilateral to nerve injury when compared to contralateral as revealed by cell counts and silver grain density. There were no significant differences between contralateral and control levels of mRNA expression as revealed by cell counts and silver grain density (Table 4.1, Figure 4.14).

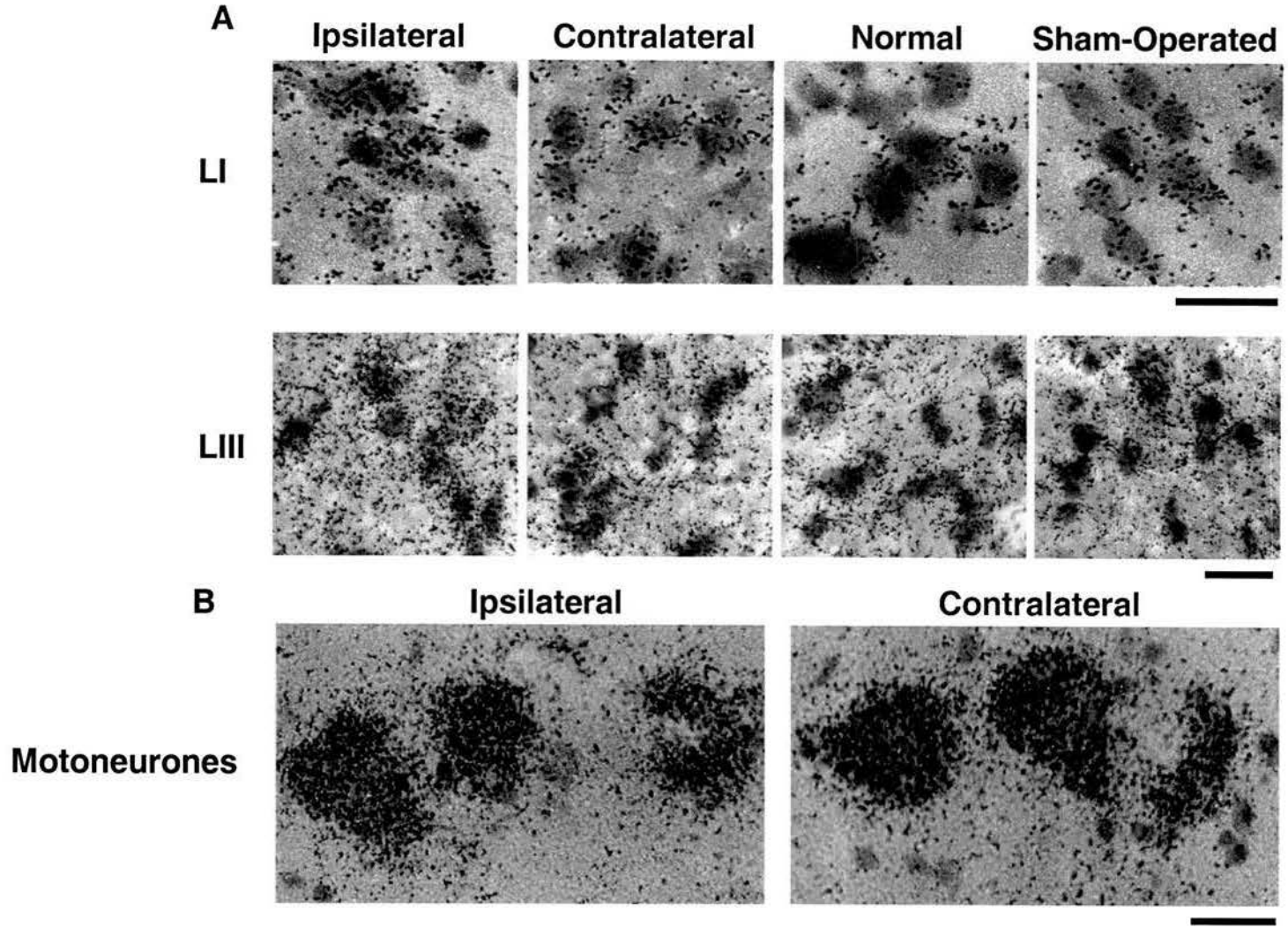

A
Ipsilateral
Contralateral
Normal
Sham-Operated
LI
LIII
B
Ipsilateral
Contralateral
Motoneurones

Table 4.1 Mean Number of Dorsal Horn Neurones Positively Expressing PKA Cα mRNA

Summary table showing the average number of dorsal horn neurones within a graticule area of 175 x 175 μm^2 positively expressing PKA Cα mRNA in lamina I, II, III, IV, and V in mediolateral and lateral locations of the dorsal horn.

Values ipsilateral to nerve injury are shown compared to contralateral, sham-operated and normal control values.

The mean number of cells expressing mRNA for PKA Cα was significantly increased ipsilateral to nerve injury in laminae I, II and III with no significant change in laminae IV and V when compared to contralateral values and to normal and sham-operated values (* $p \leq 0.05$ one way ANOVA followed by a Neuman Keul's post-hoc test). For all laminae analysed there was no significant alteration in the relative expression of Cα mRNA when comparing contralateral CCI values to normal and sham-operated values (one way ANOVA).

	Cell Counts per 175 x 175 μm^2							
	Mediolateral				Lateral			
	Ipsilateral	Contralateral	Sham	Normal	Ipsilateral	Contralateral	Sham	Normal
Lamina I	32.5 ± 3.2 *	22.5 ± 2.5	23.4 ± 2.8	21.9 ± 2.1	32.6 ± 2.3 *	23.9 ± 1.9	22.3 ± 2.6	23.1 ± 2.3
Lamina II	33.2 ± 2.2 *	22.7 ± 2.8	21.2 ± 2.6	24.1 ± 2.2	34.3 ± 2.5 *	23.4 ± 2.3	24.2 ± 2.6	23.8 ± 2.8
Lamina III	37.3 ± 2.9 *	27.3 ± 2.6	26.3 ± 3.4	25.7 ± 2.7	36.5 ± 3.2 *	29.1 ± 2.6	27.3 ± 2.7	27.3 ± 2.9
Lamina IV	43.2 ± 4.6	44.7 ± 4.1	42.6 ± 3.1	46.8 ± 4.6	38.3 ± 3.2	37.4 ± 2.4	39.1 ± 3.2	37.4 ± 3.1
Lamina V	48.1 ± 4.8	45.5 ± 4.2	46.7 ± 4.9	47.7 ± 4.6	39.1 ± 3.1	36.7 ± 3.7	40.1 ± 3.5	38.8 ± 3.8

Figure 4.14 Mean Silver Grain Density of Dorsal Horn Neurones Positively Expressing PKA Cα mRNA

Summary histogram showing the mean silver grain density for dorsal horn neurones positively expressing PKA Cα mRNA in lamina I, II, III, IV and V in mediolateral and lateral locations of the spinal cord.

Values ipsilateral to nerve injury are shown compared to contralateral, sham-operated and normal control values.

The relative silver grain density per positively expressing cell, indicative of the expression of PKA Cα mRNA, was significantly increased ipsilateral to nerve injury in lamina I, II and III but was unaltered in laminae IV and V when compared to contralateral values, normal and sham-operated values (* $p \leq 0.05$ one way ANOVA, followed by a Neuman-Keul's post-hoc test). For all laminae analysed there was no significant alteration in the relative expression of Cα mRNA when comparing contralateral CCI values to normal and sham-operated values (one way ANOVA).

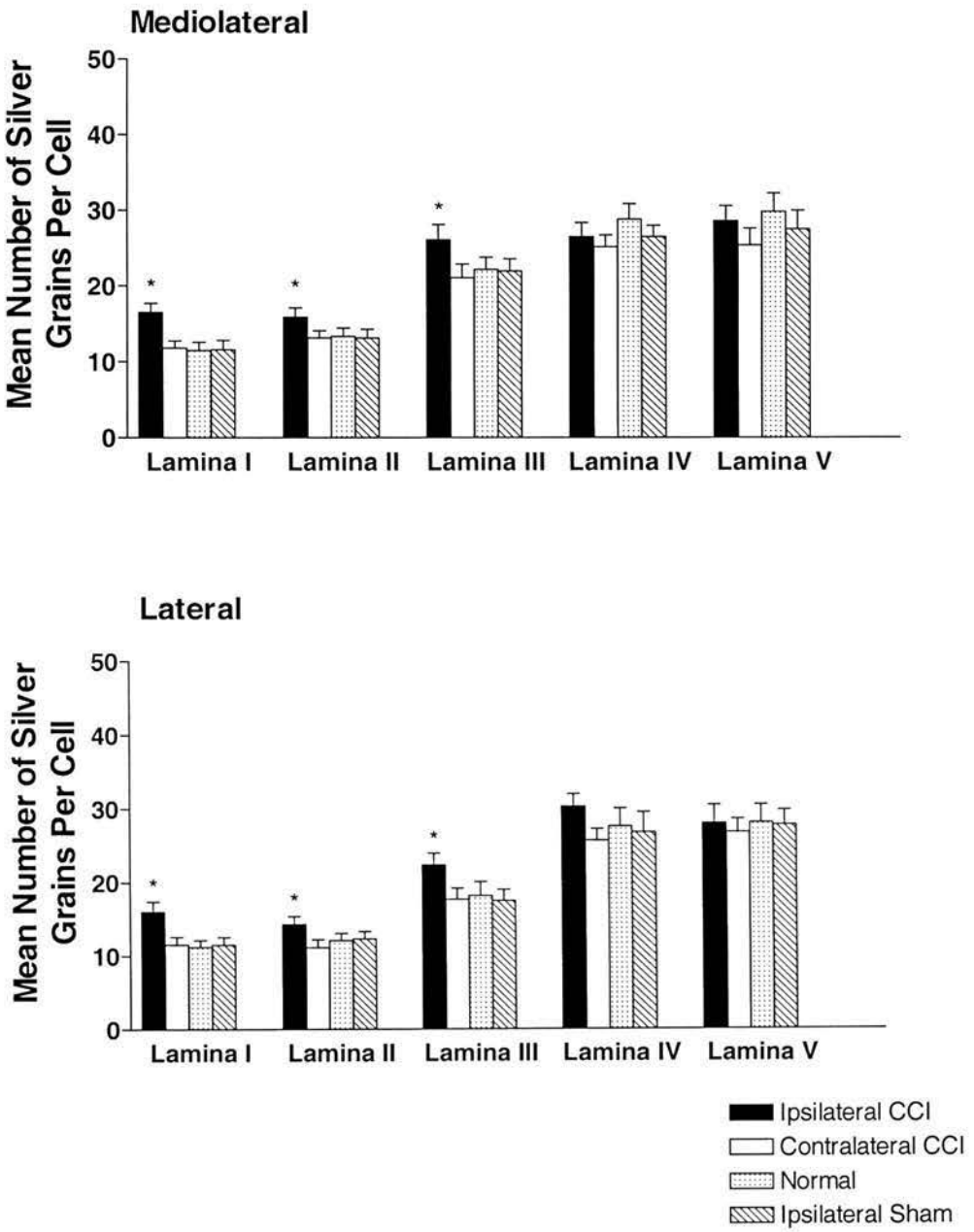

Mediolateral
Mean Number of Silver Grains Per Cell
50
40
30
20
10
0
Lamina I
Lamina II
Lamina III
Lamina IV
Lamina V
Lateral
Mean Number of Silver Grains Per Cell
50
40
30
20
10
0
Lamina I
Lamina II
Lamina III
Lamina IV
Lamina V
Ipsilateral CCI
Contralateral CCI
Normal
Ipsilateral Sham

Figure 4.15 PKA Cβ mRNA Expression in Lamina I, III and Motoneurones of the Rat Lumbar Spinal Dorsal Horn in CCI (Ipsilateral and Contralateral), Normal and Sham-operated Rats

Highpower lightfield, black and white photomicrographs showing typical levels of PKA Cβ mRNA expression in the mediolateral area of lamina I and III and motoneurones of rat lumbar spinal dorsal horn (scale bars 10μm). Photomicrographs show typical examples of the expression ipsilateral and contralateral to nerve injury and in normal and sham-operated control rats respectively. Positively labelled neurones were identified by a dense accumulation of silver grains (approximately > 5 times background expression) over and around haematoxylin stained nuclei.

Analysis of quantitative densitometry data demonstrated a significant increase in the expression of PKA Cβ subunit mRNA ipsilateral to nerve injury when compared to contralateral and control tissue in the superficial laminae of the dorsal horn (A). However, in motoneurones (B) there was no significant alteration in the expression of PKA Cβ mRNA ipsilateral to nerve injury when compared to contralateral as revealed by cell counts and silver grain density. There were no significant differences between contralateral and control levels of mRNA expression as revealed by cell counts and silver grain density (Table 4.2, Figure 4.16)

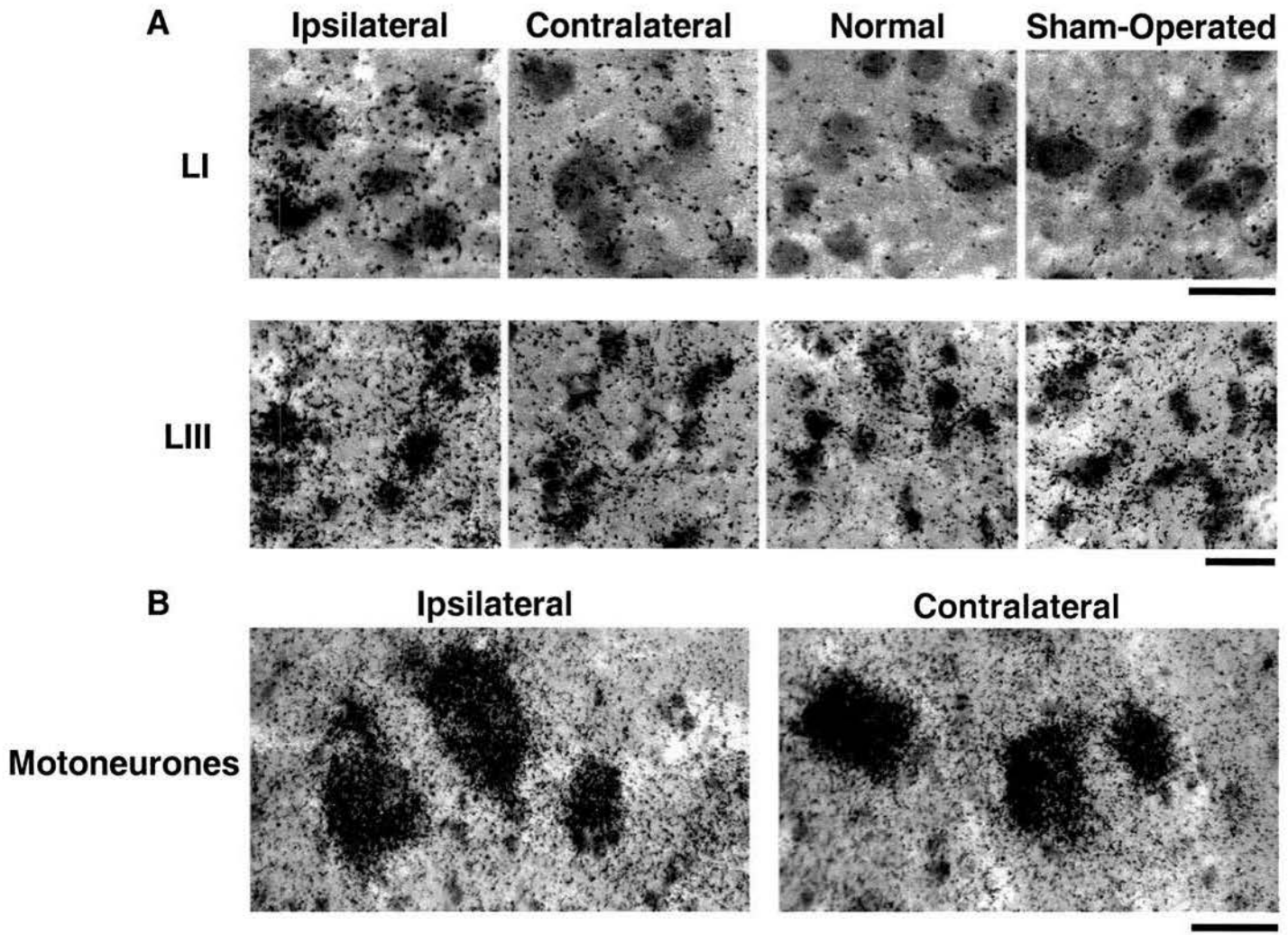

A
Ipsilateral
Contralateral
Normal
Sham-Operated
LI
LIII
B
Ipsilateral
Contralateral
Motoneurones

Table 4.2 Mean Number of Dorsal Horn Neurones Positively Expressing PKA Cβ mRNA

Summary table showing the average number of dorsal horn neurones within a graticule area of 175 x 175 μm^2 positively expressing PKA Cβ mRNA in lamina I, II, III, IV, and V in mediolateral and lateral locations of the dorsal horn.

Values ipsilateral to nerve injury are shown compared to contralateral, sham-operated and normal control values.

The mean number of cells expressing mRNA for PKA Cβ was significantly increased ipsilateral to nerve injury in laminae I, II and III compared to contralateral values and to normal and sham-operated values (* $p \leq 0.05$ one way ANOVA, followed by a Neuman-Keul's post-hoc test). For all laminae analysed there was no significant alteration in the relative expression of Cβ mRNA when comparing contralateral CCI values to normal and sham-operated values (one way ANOVA).

	Cell Counts per 175 x 175 μm^2							
	Mediolateral				Lateral			
	Ipsilateral	Contralateral	Sham	Normal	Ipsilateral	Contralateral	Sham	Normal
Lamina I	46.3 ± 4.4 *	32.2 ± 1.5	31.4 ± 2.5	28.9 ± 1.8	42.2 ± 2.4 *	34.4 ± 2.1	32.7 ± 2.5	32.5 ± 2.5
Lamina II	45.2 ± 2.8 *	31.5 ± 2.2	32.4 ± 2.6	35.5 ± 2.4	44.6 ± 2.3 *	34.2 ± 2.4	33.4 ± 2.1	34.1 ± 1.8
Lamina III	47.1 ± 1.9 *	33.5 ± 2.2	31.5 ± 3.2	32.4 ± 1.5	46.2 ± 3.8 *	32.3 ± 2.3	34.3 ± 2.1	35.3 ± 2.7
Lamina IV	52.2 ± 4.2	48.2 ± 3.2	49.2 ± 3.6	49.8 ± 4.4	50.1 ± 4.1	47.5 ± 3.4	49.7 ± 3.8	46.8 ± 3.7
Lamina V	58.9 ± 4.4	56.5 ± 3.8	58.5 ± 4.8	57.5 ± 4.2	52.3 ± 4.2	52.1 ± 4.6	50.8 ± 4.1	52.6 ± 4.5

Figure 4.16 Mean Silver Grain Density of Dorsal Horn Neurones Positively Expressing PKA Cβ mRNA

Summary histogram showing the mean silver grain density for dorsal horn neurones positively expressing PKA Cβ mRNA in lamina I, II, III, IV and V in mediolateral and lateral locations of the spinal cord.

Values ipsilateral to nerve injury are shown compared to contralateral, sham-operated and normal control values.

The relative silver grain density per positively expressing cell, indicative of the expression of PKA Cβ mRNA, was significantly increased ipsilateral to nerve injury in lamina I, II and III when compared to contralateral and values and to normal and sham-operated values (* $p \leq 0.05$ one way ANOVA, followed by a Neuman-Keuls post-hoc test). For all laminae analysed there was no significant alteration in the relative expression of PKA Cβ mRNA when comparing contralateral CCI values to normal and sham-operated values (one way ANOVA).

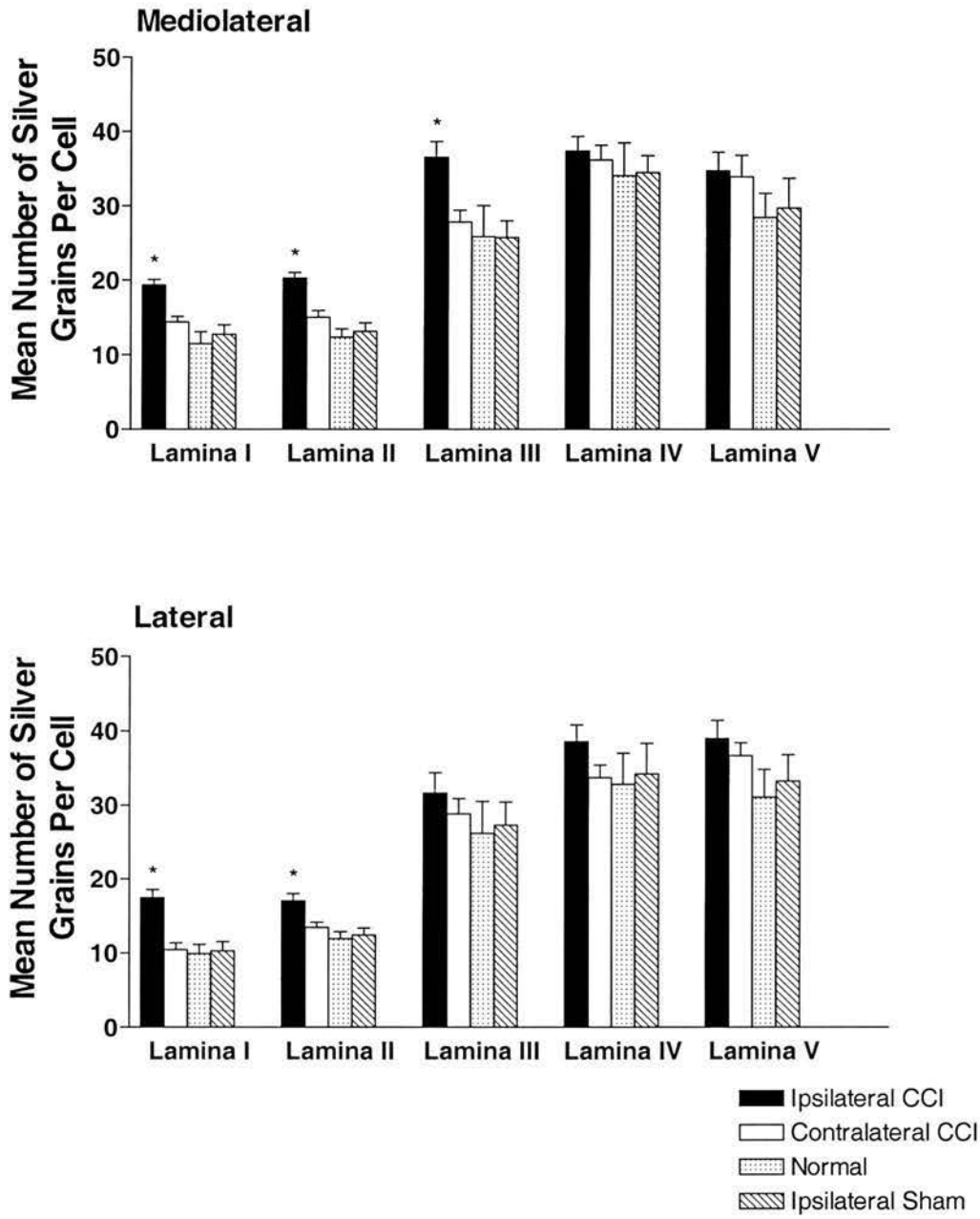

Mediolateral
Mean Number of Silver Grains Per Cell
50
40
30
20
10
0
*
*
*
Lamina I
Lamina II
Lamina III
Lamina IV
Lamina V
Lateral
Mean Number of Silver Grains Per Cell
50
40
30
20
10
0
*
*
Lamina I
Lamina II
Lamina III
Lamina IV
Lamina V
Ipsilateral CCI
Contralateral CCI
Normal
Ipsilateral Sham

Figure 4.17 PKA RIα mRNA Expression in Lamina I of the Rat Lumbar Spinal Dorsal Horn in CCI (Ipsilateral and Contralateral), Normal and Sham-Operated Rats

Highpower lightfield, black and white photomicrographs showing typical levels of PKA RIα mRNA expression in the mediolateral area of lamina I of rat lumbar spinal dorsal horn (scale bar 10μm). Photomicrographs show typical examples of the expression ipsilateral and contralateral to nerve injury and in normal and sham-operated control rats respectively. Positively labelled neurones were identified by a dense accumulation of silver grains (approximately > 5 times background expression) over and around haematoxylin stained nuclei.

Analysis of quantitative densitometry data demonstrated no significant alteration in the expression of PKA RIα mRNA ipsilateral to nerve injury when compared to contralateral and control tissue in the superficial laminae of the dorsal horn. There were also no significant differences between contralateral and control levels of mRNA expression as revealed by cell counts and silver grain density (Table 4.3, Figure 4.18).

Ipsilateral
Contralateral
Normal
Sham-Operated
LI

Table 4.3 Mean Number of Dorsal Horn Neurones Positively Expressing PKA RIα mRNA

Summary table showing the average number of dorsal horn neurones within a graticule area of 175 x 175 μm^2 positively expressing PKA RIα mRNA in lamina I, II, III, IV, and V in mediolateral and lateral locations of the dorsal horn.

Values ipsilateral to nerve injury are shown compared to contralateral, sham-operated and normal control values.

The mean number of cells expressing mRNA for PKA RIα appeared unchanged ipsilateral to nerve injury in all laminae, compared to contralateral values and to normal and sham-operated values (one way ANOVA). For all laminae analysed there was no significant alteration in the relative expression of RIα mRNA when comparing contralateral CCI values to normal and sham-operated values (one way ANOVA).

	Cell Counts per 175 x 175 μm^2							
	Mediolateral				Lateral			
	Ipsilateral	Contralateral	Sham	Normal	Ipsilateral	Contralateral	Sham	Normal
Lamina I	8.5 ± 0.6	8.1 ± 0.6	7.9 ± 1.1	8.6 ± 0.8	8.6 ± 1.1	8.8 ± 1.0	7.8 ± 0.8	8.4 ± 1.2
Lamina II	11.4 ± 1.2	11.8 ± 0.8	12.2 ± 1.8	11.4 ± 1.0	10.1 ± 1.5	10.4 ± 1.8	11.2 ± 1.6	10.8 ± 1.8
Lamina III	13.5 ± 1.4	13.2 ± 1.5	14.5 ± 1.9	12.9 ± 1.6	11.6 ± 1.2	12.1 ± 1.4	12.7 ± 1.7	11.8 ± 1.9
Lamina IV	15.5 ± 1.9	15.2 ± 2.1	16.7 ± 1.2	15.1 ± 1.6	13.5 ± 1.2	14.4 ± 1.4	14.2 ± 1.9	13.6 ± 2.1
Lamina V	15.1 ± 1.6	15.5 ± 1.2	14.5 ± 2.1	15.3 ± 1.6	13.8 ± 2.1	14.5 ± 2.4	13.9 ± 1.8	14.2 ± 1.4

Figure 4.18 Mean Silver Grain Density of Dorsal Horn Neurones Positively Expressing PKA RIα mRNA

Summary histogram showing the mean silver grain density for dorsal horn neurones positively expressing PKA RIα mRNA in lamina I, II, III, IV and V in mediolateral and lateral locations of the spinal cord.

Values ipsilateral to nerve injury are shown compared to contralateral, sham-operated and normal control values.

The relative silver grain density per positively expressing cell, indicative of the expression of PKA RIα mRNA, was unchanged ipsilateral to nerve injury in all laminae when compared to contralateral values and normal and sham-operated values (* $p \leq 0.05$ one way ANOVA, followed by a Neuman-Keul's post-hoc test). For all laminae analysed there was no significant alteration in the relative expression of PKA RIα mRNA when comparing contralateral CCI values to normal and sham-operated values (one way ANOVA) compared to contralateral values (paired Student's t-test).

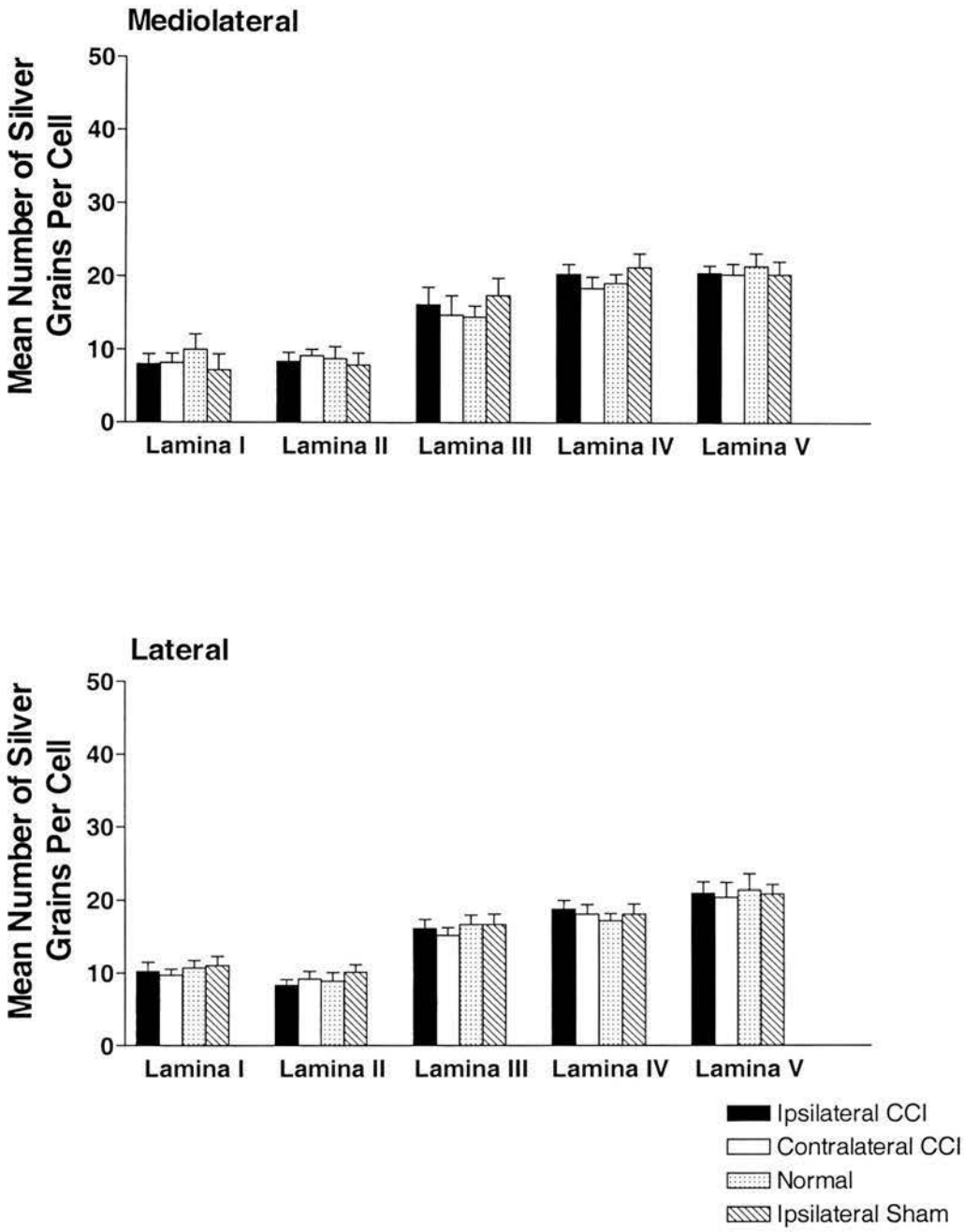

Mediolateral
Mean Number of Silver Grains Per Cell
50
40
30
20
10
0
Lamina I
Lamina II
Lamina III
Lamina IV
Lamina V
Lateral
Mean Number of Silver Grains Per Cell
50
40
30
20
10
0
Lamina I
Lamina II
Lamina III
Lamina IV
Lamina V
Ipsilateral CCI
Contralateral CCI
Normal
Ipsilateral Sham

Figure 4.19 PKA RIβ mRNA Expression in Lamina I of the Rat Lumbar Spinal Dorsal Horn in CCI (Ipsilateral and Contralateral), Normal and Sham-Operated Rats

Highpower lightfield, black and white photomicrographs showing typical levels of PKA RIβ mRNA expression in the mediolateral area of lamina I of rat lumbar spinal dorsal horn (scale bar 10μm). Photomicrographs show typical examples of the expression ipsilateral and contralateral to nerve injury and in normal and sham-operated control rats respectively. Positively labelled neurones were identified by a dense accumulation of silver grains (approximately > 5 times background expression) over and around haematoxylin stained nuclei.

Analysis of quantitative densitometry data demonstrated no significant alteration in the expression of PKA RIβ mRNA ipsilateral to nerve injury when compared to contralateral and control tissue in the superficial laminae of the dorsal horn. There were also no significant differences between contralateral and control levels of mRNA expression as revealed by cell counts and silver grain density (Table 4.4, Figure 4.20).

Ipsilateral
Contralateral
Normal
Sham-Operated
LI

Table 4.4 Mean Number of Dorsal Horn Neurones Positively Expressing PKA RIβ mRNA

Summary table showing the average number of dorsal horn neurones within a graticule area of 175 x 175 μm^2 positively expressing PKA RIβ mRNA in lamina I, II, III, IV, and V in mediolateral and lateral locations of the dorsal horn.

Values ipsilateral to nerve injury are shown compared to contralateral, sham-operated and normal control values.

The mean number of cells expressing mRNA for PKA RIβ appeared unchanged ipsilateral to nerve injury in all laminae when compared to contralateral values and to normal and sham-operated values (one way ANOVA). For all laminae analysed there was no significant alteration in the relative expression of PKA RIβ mRNA when comparing contralateral CCI values to normal and sham-operated values (one way ANOVA).

	Cell Counts per 175 x 175 μm^2							
	Mediolateral				Lateral			
	Ipsilateral	Contralateral	Sham	Normal	Ipsilateral	Contralateral	Sham	Normal
Lamina I	8.3 ± 0.8	8.6 ± 0.7	8.9 ± 0.8	8.1 ± 0.7	8.1 ± 0.8	9.1 ± 1.2	8.5 ± 1.2	8.6 ± 1.0
Lamina II	13.4 ± 1.2	12.8 ± 1.3	13.2 ± 1.4	12.6 ± 1.5	9.9 ± 1.1	10.1 ± 1.2	10.4 ± 1.1	10.2 ± 1.4
Lamina III	16.4 ± 1.6	15.5 ± 1.2	16.5 ± 1.7	15.9 ± 1.5	10.6 ± 1.2	11.1 ± 1.5	11.5 ± 1.4	11.2 ± 1.3
Lamina IV	16.5 ± 2.1	15.8 ± 1.7	16.2 ± 1.9	15.8 ± 1.7	11.0 ± 1.1	11.4 ± 1.4	11.3 ± 1.3	12.5 ± 1.7
Lamina V	15.5 ± 1.4	15.1 ± 1.7	16.1 ± 1.9	15.8 ± 1.4	11.2 ± 1.8	12.5 ± 2.1	11.9 ± 1.6	11.7 ± 1.7

Figure 4.20 Mean Silver Grain Density of Dorsal Horn Neurones Positively Expressing PKA RIβ mRNA

Summary histogram showing the mean silver grain density for dorsal horn neurones positively expressing PKA RIβ mRNA in lamina I, II, III, IV and V in mediolateral and lateral locations of the spinal cord.

Values ipsilateral to nerve injury are shown compared to contralateral, sham-operated and normal control values.

The relative silver grain density per positively expressing cell, indicative of the expression of PKA RIβ mRNA, was unchanged ipsilateral to nerve injury in all laminae when compared to contralateral values and normal and sham-operated values (one way ANOVA). For all laminae analysed there was no significant alteration in the relative expression of PKA RIβ mRNA when comparing contralateral CCI values to normal and sham-operated values (one way ANOVA).

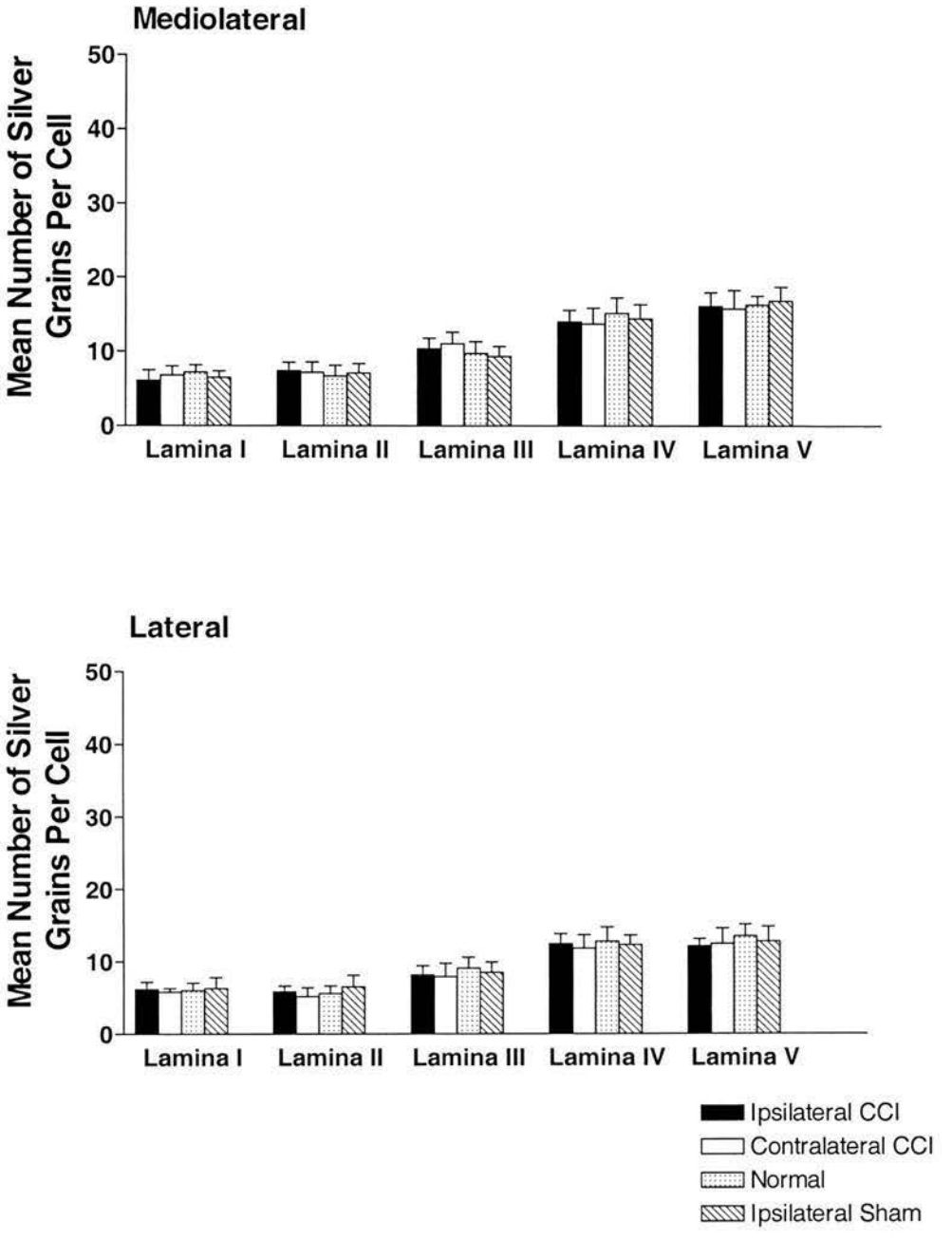

Mediolateral
Mean Number of Silver Grains Per Cell
50
40
30
20
10
0
Lamina I
Lamina II
Lamina III
Lamina IV
Lamina V
Lateral
Mean Number of Silver Grains Per Cell
50
40
30
20
10
0
Lamina I
Lamina II
Lamina III
Lamina IV
Lamina V
Ipsilateral CCI
Contralateral CCI
Normal
Ipsilateral Sham

Figure 4.21 PKA RIIα mRNA Expression in Lamina I of the Rat Lumbar Spinal Dorsal Horn in CCI (Ipsilateral and Contralateral), Normal and Sham-Operated Rats

Highpower lightfield, black and white photomicrographs showing typical levels of PKA RIIα mRNA expression in the mediolateral area of lamina I of rat lumbar spinal dorsal horn (scale bar 10μm). Photomicrographs show typical examples of the expression ipsilateral and contralateral to nerve injury and in normal and sham-operated control rats respectively. Positively labelled neurones were identified by a dense accumulation of silver grains (approximately > 5 times background expression) over and around haematoxylin stained nuclei.

Analysis of quantitative densitometry data demonstrated no significant alteration in the expression of PKA RIIα mRNA ipsilateral to nerve injury when compared to contralateral and control tissue in the superficial laminae of the dorsal horn. There were also no significant differences between contralateral and control levels of mRNA expression as revealed by cell counts and silver grain density (Table 4.5, Figure 4.22).

Ipsilateral
Contralateral
Normal
Sham-Operated
LI

Table 4.5 Mean Number of Dorsal Horn Neurones Positively Expressing PKA RIIα mRNA

Summary table showing the average number of dorsal horn neurones within a graticule area of 175 x 175 μm^2 positively expressing PKA RIIα mRNA in lamina I, II, III, IV, and V in mediolateral and lateral locations of the dorsal horn.

Values ipsilateral to nerve injury are shown compared to contralateral, sham-operated and normal control values.

The mean number of cells expressing mRNA for PKA RIIα appeared unchanged ipsilateral to nerve injury in all laminae when compared to contralateral values and normal and sham-operated values (one way ANOVA). For all laminae analysed there was no significant alteration in the relative expression of PKA RIIα mRNA when comparing contralateral CCI values to normal and sham-operated values (one way ANOVA).

	Cell Counts per 175 x 175 μm^2							
	Mediolateral				Lateral			
	Ipsilateral	Contralateral	Sham	Normal	Ipsilateral	Contralateral	Sham	Normal
Lamina I	18.2 ± 1.9	19.5 ± 1.6	18.1 ± 1.8	18.7 ± 1.6	16.5 ± 1.2	15.4 ± 1.8	15.2 ± 1.7	15.7 ± 1.5
Lamina II	22.4 ± 2.0	23.2 ± 1.8	23.1 ± 2.3	22.1 ± 1.9	21.5 ± 2.0	22.1 ± 1.9	22.9 ± 1.4	21.2 ± 1.6
Lamina III	25.2 ± 2.5	24.1 ± 2.6	25.1 ± 1.9	23.9 ± 2.8	24.2 ± 1.4	25.1 ± 2.1	24.7 ± 2.2	24.8 ± 1.7
Lamina IV	25.5 ± 2.4	24.5 ± 2.1	24.2 ± 2.6	25.1 ± 2.1	24.3 ± 2.2	25.5 ± 1.9	25.8 ± 2.0	24.8 ± 1.9
Lamina V	25.7 ± 2.3	24.9 ± 2.6	24.8 ± 2.7	24.5 ± 2.4	25.5 ± 2.0	25.9 ± 2.4	25.1 ± 1.9	26.1 ± 2.4

Figure 4.22 Mean Silver Grain Density of Dorsal Horn Neurones Positively Expressing PKA RIIα mRNA

Summary histogram showing the mean silver grain density for dorsal horn neurones positively expressing PKA RIIα mRNA in lamina I, II, III, IV and V in mediolateral and lateral locations of the spinal cord.

Values ipsilateral to nerve injury are shown compared to contralateral, sham-operated and normal control values.

The relative silver grain density per positively expressing cell, indicative of the expression of PKA RIIα mRNA, was unchanged ipsilateral to nerve injury in all laminae when compared to contralateral values and to normal and sham-operated values (one way ANOVA). For all laminae analysed there were no significant alteration in the relative expression of PKA RIIα mRNA when comparing contralateral CCI values to normal and sham-operated values (one way ANOVA).

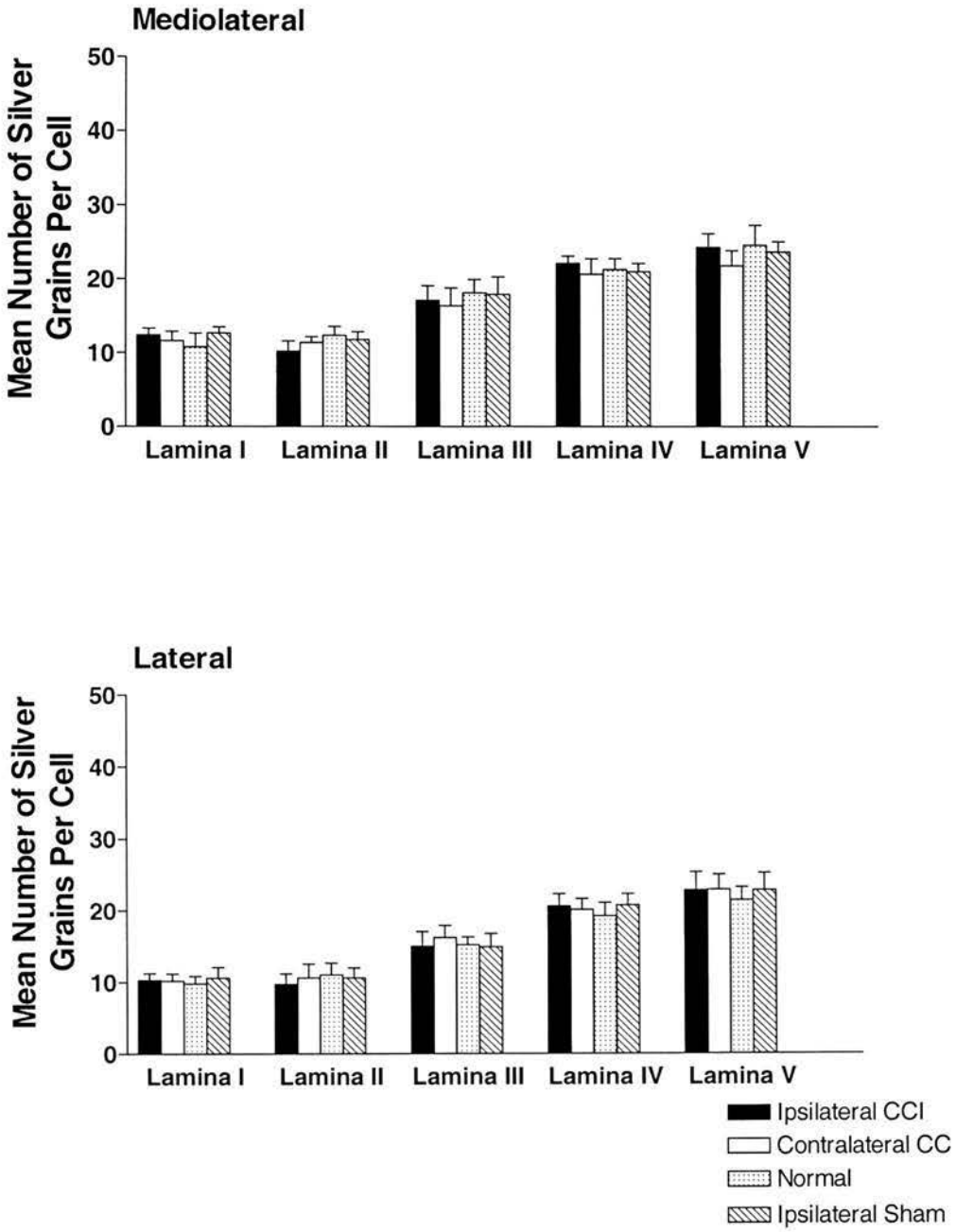

Mediolateral
Mean Number of Silver Grains Per Cell
50
40
30
20
10
0
Lamina I
Lamina II
Lamina III
Lamina IV
Lamina V
Lateral
Mean Number of Silver Grains Per Cell
50
40
30
20
10
0
Lamina I
Lamina II
Lamina III
Lamina IV
Lamina V
Ipsilateral CCI
Contralateral CC
Normal
Ipsilateral Sham

Figure 4.23 PKA RIIβ mRNA Expression in Lamina I, of the Rat Lumbar Spinal Dorsal Horn in CCI (Ipsilateral and Contralateral), Normal and Sham-Operated Rats

Highpower lightfield, black and white photomicrographs showing typical levels of PKA RIIβ mRNA expression in the mediolateral area of lamina I of rat lumbar spinal dorsal horn (scale bar 10μm). Photomicrographs show typical examples of the expression ipsilateral and contralateral to nerve injury and in normal and sham-operated control rats respectively. Positively labelled neurones were identified by a dense accumulation of silver grains (approximately > 5 times background expression) over and around haematoxylin stained nuclei.

Analysis of quantitative densitometry data demonstrated no significant alteration in the expression of PKA RIIβ mRNA ipsilateral to nerve injury when compared to contralateral and control tissue in the superficial laminae of the dorsal horn. There was also no significant differences between contralateral and control levels of mRNA expression as revealed by cell counts and silver grain density (Table 4.6, Figure 4.24).

Ipsilateral
Contralateral
Normal
Sham-Operated
LI

Table 4.6 Mean Number of Dorsal Horn Neurones Positively Expressing PKA RIIβ mRNA

Summary table showing the average number of dorsal horn neurones within a graticule area of 175 x 175 μm^2 positively expressing PKA RIIβ mRNA in lamina I, II, III, IV, and V in mediolateral and lateral locations of the dorsal horn.

Values ipsilateral to nerve injury are shown compared to contralateral, sham-operated and normal control values.

The mean number of cells expressing mRNA for PKA RIIβ appeared unchanged ipsilateral to nerve injury in all laminae when compared to contralateral values and to normal and sham-operated values (one way ANOVA). For all laminae analysed there was no significant alteration in the relative expression of PKA RIIβ mRNA when comparing contralateral CCI values to normal and sham-operated values (one way ANOVA).

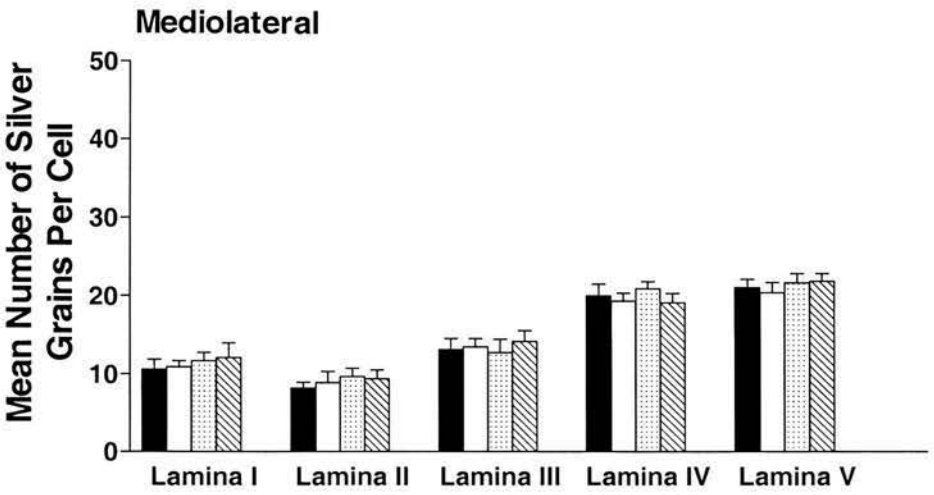
	Cell Counts per 175 x 175 μm^2							
	Mediolateral				Lateral			
	Ipsilateral	Contralateral	Sham	Normal	Ipsilateral	Contralateral	Sham	Normal
Lamina I	13.3 ± 1.5	13.5 ± 1.9	14.2 ± 1.8	13.7 ± 1.7	12.2 ± 1.1	12.4 ± 1.6	12.7 ± 1.6	11.9 ± 1.7
Lamina II	15.4 ± 1.9	16.2 ± 1.8	15.2 ± 1.9	16.1 ± 1.7	14.6 ± 1.7	14.1 ± 1.4	15.2 ± 1.9	15.2 ± 1.6
Lamina III	16.4 ± 1.9	16.9 ± 1.5	17.4 ± 1.8	16.9 ± 1.6	15.3 ± 1.9	16.1 ± 2.1	16.5 ± 1.9	15.2 ± 1.8
Lamina IV	18.5 ± 2.4	18.4 ± 1.9	19.2 ± 2.1	18.6 ± 1.8	18.0 ± 1.9	17.5 ± 1.6	17.3 ± 1.4	17.5 ± 1.7
Lamina V	17.5 ± 1.9	17.1 ± 2.3	17.4 ± 1.9	18.2 ± 1.7	17.4 ± 1.4	17.7 ± 2.0	17.9 ± 1.7	16.7 ± 1.8

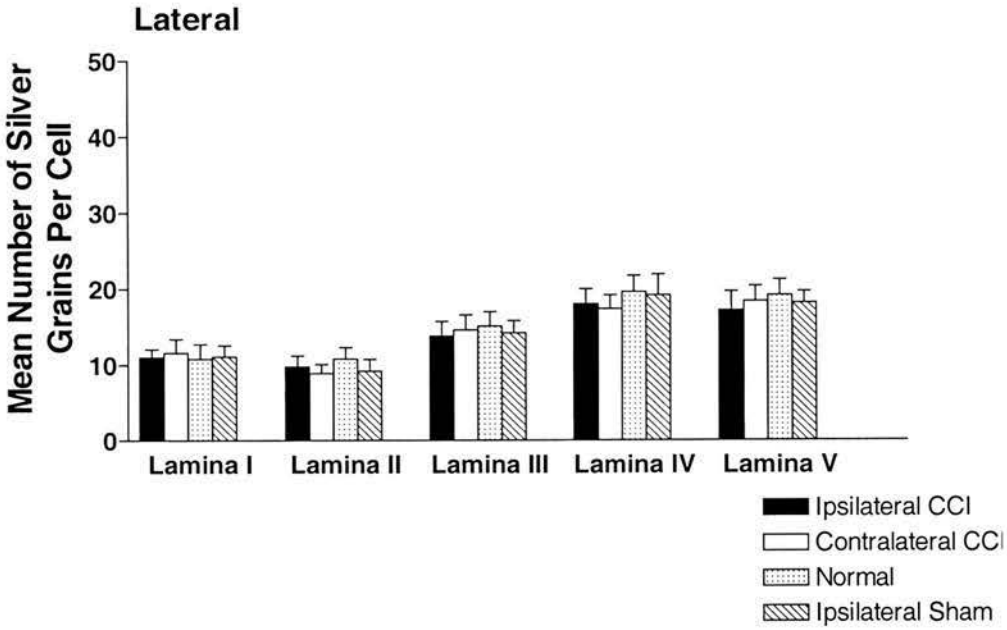
Figure 4.24 Mean Silver Grain Density of Dorsal Horn Neurones Positively Expressing PKA RIIβ mRNA

Summary histogram showing the mean silver grain density for dorsal horn neurones positively expressing PKA RIIβ mRNA in lamina I, II, III, IV and V in mediolateral and lateral locations of the spinal cord.

Values ipsilateral to nerve injury are shown compared to contralateral, sham-operated and normal control values.

The relative silver grain density per positively expressing cell, indicative of the expression of PKA RIIβ mRNA, was unchanged ipsilateral to nerve injury in all laminae when compared to contralateral values and to normal and sham-operated values (one way ANOVA). For all laminae analysed there was no significant alteration in the relative expression of PKA RIIβ mRNA when comparing contralateral CCI values to normal and sham-operated values (one way ANOVA).


Mediolateral
Mean Number of Silver Grains Per Cell
50
40
30
20
10
0
Lamina I
Lamina II
Lamina III
Lamina IV
Lamina V


Lateral
Mean Number of Silver Grains Per Cell
50
40
30
20
10
0
Lamina I
Lamina II
Lamina III
Lamina IV
Lamina V
Ipsilateral CCI
Contralateral CCI
Normal
Ipsilateral Sham

4.5.3 Determination of Protein Levels of Catalytic Subunits and Regulatory Subunits of cAMP Dependent Protein Kinase A (PKA) Following CCI

4.5.3.1 Catalytic Subunits of PKA (Cα and Cβ)

In order to determine whether the increase in the expression of mRNA for the catalytic subunits of PKA leads to increased steady state levels of catalytic subunit protein in the lumbar spinal cord following CCI, Western blot analysis was carried out using an antibody which recognises both catalytic subunits of PKA (PKA Cα/β; Table 2.1). These results are based on data obtained from a total of 12 rats (n=4 CCI, n=4 Sham and n=4 normal). Using materials and methods described in detail in the general procedures chapter (Chapter 2 section 2.3.8), western blots were carried out using the catalytic subunit specific antibody and an antibody specific to the established cellular housekeeping enzyme GAPDH (Table 2.1). Samples were normalised in relation to protein levels and to GAPDH expression and values for Cα/β immunoreactivity were expressed as the percentage GAPDH expression (Figure 4.25).

Quantitative densitometry of immunoreactive bands revealed an increase in the levels of both Cα and Cβ in the spinal cord ipsilateral to nerve injury when compared to contralateral, sham-operated and normal values (*$p \leq 0.05$ Student's unpaired t-test; Figure 4.25).

4.5.3.2 Regulatory Subunits of PKA (RIα, RIβ, RIIα and RIβ)

In order to determine whether there was any alteration in the levels of protein for the regulatory subunits of PKA in the lumbar spinal cord following CCI, Western blot analysis was carried out using several antibodies. An antibody which recognises both type I regulatory subunits of PKA (RIα and RIβ), (PKA-RI), an antibody which specifically recognises RIIβ (PKA-RIIβ) and an antibody specific to RIIα (PKA RIIα) (Table 2.1). These results were obtained from a total of 36 rats (n=12 CCI, n=12 sham-operated and n=12 normal). Using materials and methods described

previously (Chapter2 section 2.3.8) western blots were carried out using the specific antibodies and an antibody specific to GAPDH. Samples were normalised in relation to GAPDH and values expressed as the percentage GAPDH expression (Figure 4.26).

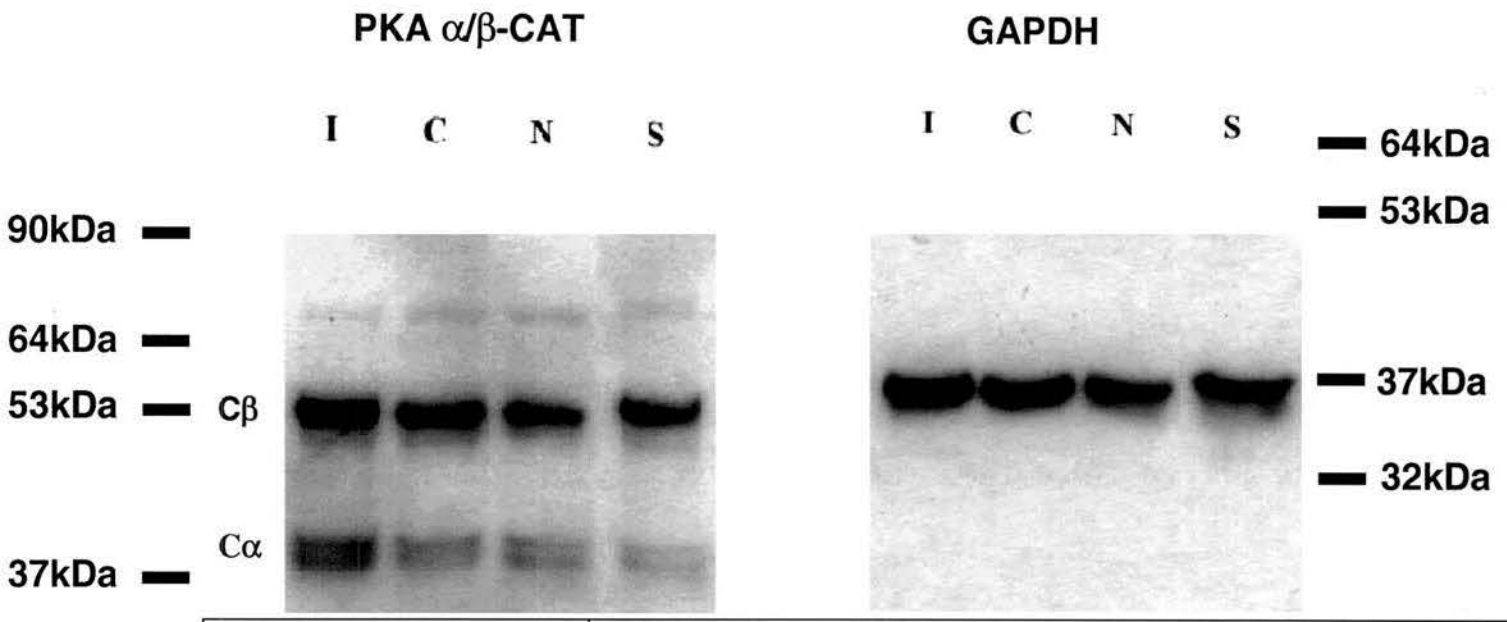
Quantitative densitometry of immunoreactive bands revealed a contralateral decrease in the level of RIβ subunit protein when compared to normal and sham-operated values ($*p \leq 0.05$ Student's unpaired t-test; Figure 4.26) and no alterations in the levels of protein for RIIα or RIIβ in the lumbar spinal cord following CCI surgery (Student's unpaired t-test; Figure 4.27 and 4.28).

Figure 4.25 Western Blot Analysis of PKA Catalytic Subunits (Cα and Cβ) and GAPDH Protein Expression in the Rat Lumbar Spinal Cord in CCI (Ipsilateral and Contralateral), Normal and Sham-Operated Rats

(A) Western blots of hemisected lumbar spinal cord tissue showing Cα (42kDA) and Cβ (53kDa) protein expression and the housekeeping protein GAPDH (36kDa). Scanner print-outs from ECL films show typical examples of Cα, Cβ and GAPDH expression within the same lanes. The positions of the molecular weight markers are shown.

The expression of both Cα and Cβ protein ipsilateral to nerve injury (I) appeared consistently greater than that in contralateral (C), normal (N) and sham-operated (S) tissue.

(B) Table represents Cα and Cβ expression as a percentage of GAPDH expression in terms of relative grey scale values following quantitative densitometry of ECL films. Data are presented as mean ± SEM (n=4) (* $p \leq 0.05$, compared to contralateral, sham-operated and normal rats Student's unpaired t-test).



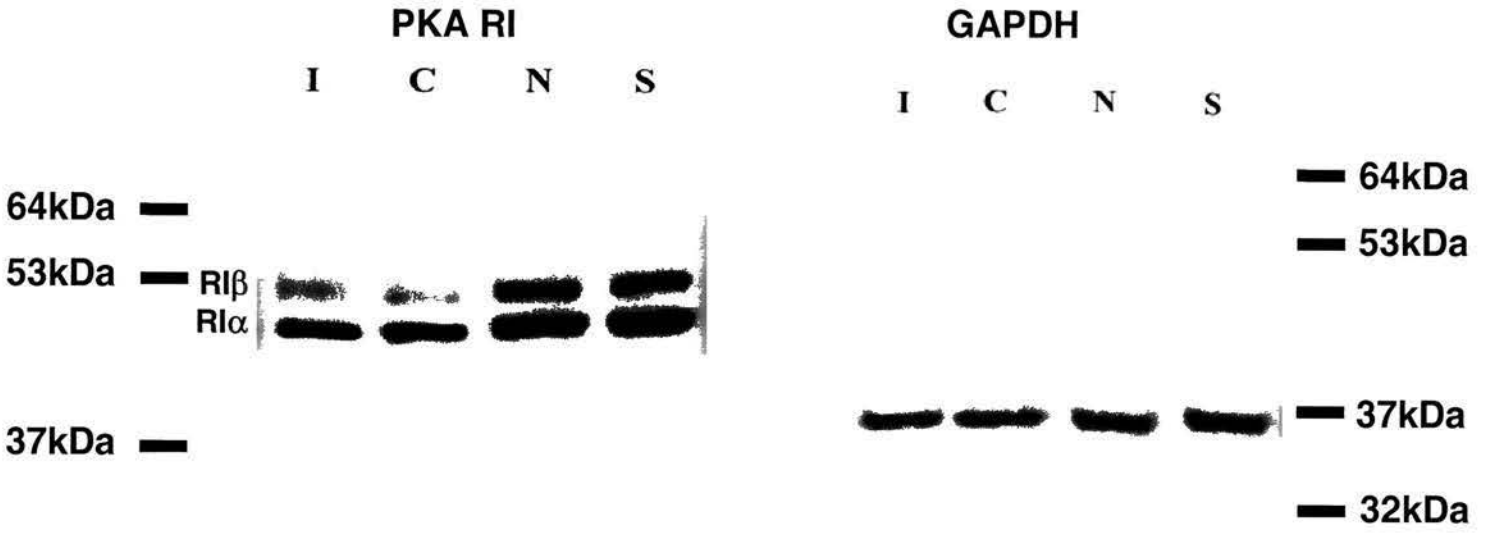
		PKA (Cα/β) Immunoreactivity (% of GAPDH)			
		Ipsilateral	Contralateral	Normal	Sham
PKA Cα	Mean ± SEM	77.3 ± 12.6*	50.4 ± 13.5	46.2 ± 7.3	45.6 ± 12.9
PKA Cβ	Mean ± SEM	32.4 ± 6.7*	21.4 ± 4.6	18.3 ± 5.3	16.9 ± 8.9

Figure 4.26 PKA (RIα and RIβ), and GAPDH Protein Expression in the Rat Lumbar Spinal Cord in CCI (Ipsilateral and Contralateral), Normal and Sham-Operated Rats

(A) Western blots of hemisected lumbar spinal cord tissue showing RIα (46kDa), RIβ (52kDa) protein expression and the housekeeping protein GAPDH (36kDa). Scanner print-outs from the ECL films show typical examples of RIα, RIβ and GAPDH expression within the same lanes. The positions of the molecular weight markers are shown.

The expression of RIβ protein in CCI tissue ipsilateral (I) and contralateral (C) to nerve injury appeared consistently decreased than that in normal (N) and sham-operated (S) tissue. The expression of RIα protein ipsilateral to nerve injury (I) appeared unaltered when compared to that in contralateral (C), normal (N) and sham-operated (S) tissue.

(B) Table represents RIα and RIβ expression as a percentage of GAPDH expression in terms of relative grey scale values following quantitative densitometry of ECL films. Data are presented as mean ± SEM (n=4) (*$p \leq 0.05$ compared to contralateral, sham-operated and normal rats Student's unpaired t-test).



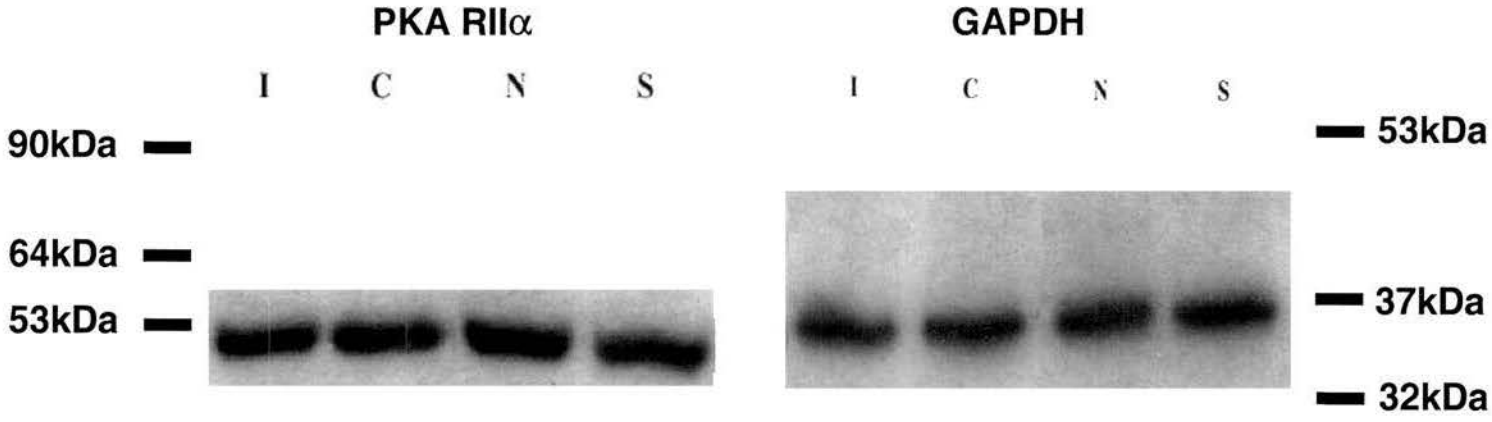
		PKA RI (α/β) Immunoreactivity (% of GAPDH)			
		Ipsilateral	Contralateral	Normal	Sham
PKA RIα	Mean ± SEM	148.8 ± 21.9	115.2 ±18.6	141.3 ±17.8	124.6 ± 20.0
PKA RIβ	Mean ± SEM	45.9 ± 10.7*	39.9 ± 12.2*	60.3 ± 12.7	56.4 ± 9.9

Figure 4.27 Western Blot Analysis of RIIα and GAPDH Protein Expression in the Rat Lumbar Spinal Cord in CCI (Ipsilateral and Contralateral), Normal and Sham-Operated Rats

(A) Western blots of hemisected lumbar spinal cord tissue showing RIIα (51kDa) protein expression and the housekeeping protein GAPDH (36kDa). Scanner print-outs from the ECL films show typical examples of RIIα and GAPDH expression within the same lanes. The positions of the molecular weight markers are shown.

The expression of RIIα protein ipsilateral to nerve injury (I) appeared unaltered when compared to that in contralateral (C), normal (N) or sham-operated (S) tissue.

(B) Table represents RIIα expression as a percentage of GAPDH expression in terms of relative grey scale values following quantitative densitometry of ECL films. Data are presented as mean ± SEM (n=4, Student's unpaired t-test).



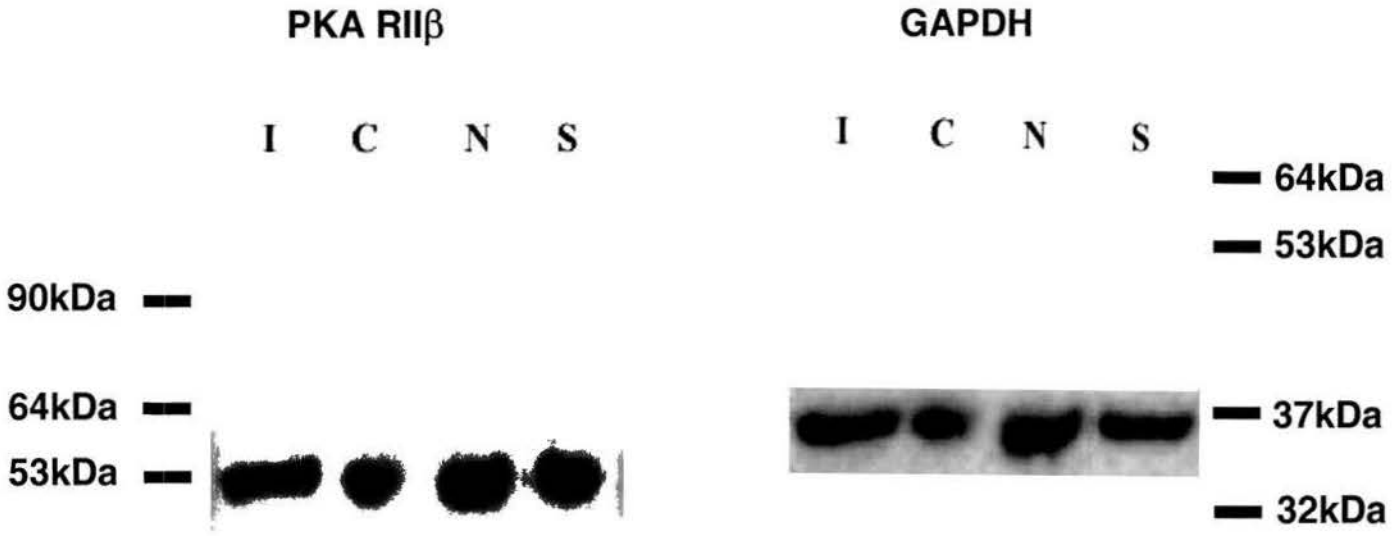
	PKA RIIα Immunoreactivity (% of GAPDH)			
	Ipsilateral	Contralateral	Normal	Sham
Mean ± SEM	134.6 ±1.2	134.8 ±1.0	130.8 ±3.2	136.8 ± 3.6

Figure 4.28 RIIβ and GAPDH Protein Expression in the Rat Lumbar Spinal Cord in CCI (Ipsilateral and Contralateral) Normal and Sham-Operated Rats

(A) Western blots of hemisected lumbar spinal cord tissue showing RIIβ (53kDa) protein expression and the housekeeping protein GAPDH (36kDa). Scanner print-outs from the ECL films show typical examples of RIIβ and GAPDH expression within the same lanes. The positions of the molecular weight markers are shown.

The expression of RIIβ protein ipsilateral to nerve injury (I) appeared unaltered when compared to that in contralateral (C), normal (N) or sham-operated (S) tissue.

(B) Table represents RIIβ expression as a percentage of GAPDH expression in terms of relative grey scale values following quantitative densitometry of ECL films. Data are presented as mean ± SEM (n=4, Student's unpaired t-test).



	PKA RIIβ Immunoreactivity (% of GAPDH)			
	Ipsilateral	Contralateral	Normal	Sham
Mean ± SEM	101.5 ± 36.1	86.56 ± 26.72	103.25 ± 34.89	112.13 ± 30.11

4.6 Discussion

There is considerable evidence that the underlying mechanisms of persistent pain after nerve injury result from long-term changes in nociceptive processing of peripheral sensory neurones (peripheral sensitisation) and/or neurones of the CNS (central sensitisation). Many studies have focused on NMDA mediated long-term changes in the firing of dorsal horn neurones (eg. Woolf and Thompson, 1991). However, while ionotropic receptors may contribute to the initiation of peripheral and central sensitisation, it seems likely that second messenger-activated changes in neuronal activity account for the maintenance of these conditions. The behaviour of all cells is governed by signalling systems that translate external information into a repertoire of internal signals (eg. second messenger systems and their downstream targets, such as voltage-gated and ligand-gated ion-channels and gene expression). Studies have provided evidence for a contribution of specific second messenger pathways to the establishment of these prolonged changes in the excitability of dorsal horn neurones (Coderre et al., 1993).

Peripheral sensitisation may be evoked by a variety of inflammatory mediators. There is substantial evidence that the mechanism of prostaglandin-induced sensitisation of sensory neurones involves activation of the cAMP transduction casade (Taiwo et al., 1989; Taiwo and Levine, 1991; Hintgen et al., 1995; England et al., 1996; Kress et al., 1996; Malmberg et al., 1997). At the spinal cord level, several studies have demonstrated that the persistent pain states after nerve injury are sensitive to antagonists selective for the NMDA subtype of glutamate receptors (Coderre and Melzack, 1992; Nasstrom et al., 1992; Chaplan et al., 1997). The NMDA receptor is highly permeable to Ca^{2+} and several second messenger activated pathways have been implicated in NMDA receptor signalling. After stimulation, the second messenger-activated protein kinases may phosphorylate various substrate proteins such as ion channels, G-protein coupled receptors and other enzymes, to enhance neuronal function and pain signalling. Specifically, PKC and PKA have been implicated in the establishment of changes in excitability of neurones involved in nociceptive transmission (Taiwo et al., 1989; Taiwo and Levine, 1991; Cerne et

al., 1992, 1993; Mao et al., 1993; Palecek et al., 1994; Lin et al., 1996; Sluka et al., 1997; Malmberg et al., 1997)

Intrathecal injection of the PKA inhibitors H89 and myr-PKI (5-24) were carried out to evaluate the contribution of the catalytic subunits of PKA to the maintenance of the abnormal behavioural responses that occur following CCI. H89 and myr-PKI (5-24) specifically inhibit the catalytic subunits of PKA whilst Rp-cAMPs is a competitive inhibitor of the activation of PKA by cAMP and allows us to identification of the contribution of cAMP to PKA regulation following CCI. In rats at the peak of neuropathy intrathecal injection of H89, myr-PKI (5-24) and RpcAMPs significantly reversed the abnormal behavioural manifestations that develop following CCI and are believed to be indicative of neuropathic pain. This inhibition was specific to the ipsilateral limb and the agents showed no effect on the contralateral limb or baseline values in normal unoperated rats. Control intrathecal injection of vehicle and an inactive myristoylated peptide in CCI rats also had no effect. These results highlight the potential contribution of PKA catalytic activity to the abnormal behavioural responses that occur following nerve injury. They also demonstrate that the activation of PKA by cAMP makes a contribution. Thus cAMP-regulated PKA activity and potentially also unregulated PKA catalytic activity may contribute to central sensitisation. The fact that the agents can reverse established sensitisation suggests that an active dynamic phosphorylation of key target substrates by enzymatically active PKA is likely to be an important component in maintaining central sensitisation.

In situ hybridisation histochemistry and Western blot experiments were carried out to investigate the levels of catalytic and regulatory subunits of PKA and determine any alterations in the levels of expression of mRNA and protein in the dorsal horn of the spinal cord. The levels of expression of mRNA and protein for both PKA catalytic subunits (Cα and Cβ) were unilaterally increased in the ipsilateral dorsal horn, specifically in the superficial laminae (LI, II and III). The superficial laminae are regions where primary afferent Aδ and C-polymodal nociceptors predominantly terminate. The expression of mRNA for the regulatory subunits of PKA (RIα, RIβ,

RIIα and RIIβ) remained unaltered following CCI. However, Western blot analysis demonstrated a decrease of RIβ protein levels following CCI. Interestingly it appears from the results that as well as the increase in the catalytic subunits of PKA within the superficial dorsal horn, which would presumably lead to an increase in the unrestrained catalytic activity of PKA within these dorsal horn neurones, there was also a decrease in the protein levels of the neurospecific isoform of a PKA regulatory subunit RIβ. Presumably this would lead to a further, and neurospecific imbalance in the inhibitory regulatory activity of PKA leading to a further increase in the levels of unrestrained catalytic activity following CCI. The altered expression of selective genes and protein products of PKA within this region of the dorsal horn suggests that prolonged functional changes in PKA activity may occur centrally following CCI. This apparent increase in PKA catalytic activity due to an alteration of the R:C ratio may contribute to the abnormal behavioural responses following CCI and together with the observations that spinally administered inhibitors of PKA can substantially reverse these abnormal behavioural responses suggests that the sustained activation of PKA may be an integral mechanism underlying central sensitisation following CCI.

These findings are consistent with other studies suggesting that PKA plays a role in prolonged changes in neuronal synaptic efficacy, which include studies of long-term potentiation (LTP) in the hippocampus (Frey et al., 1993; Huang et al., 1996) and long-term facilitation (LTF) in *Aplysia* (Byrne and Kandel, 1996; Kandel and Schwartz, 1982). The induction of LTP is presently thought to require both activation of NMDA receptors by synaptically released glutamate (Collingridge et al., 1983) and depolarization of the postsynaptic membrane (Gustafsson and Wigstrom, 1986). The biochemical cascades that lead to the associated form of LTP appear to be triggered by a surge of free Ca^{2+} ions in the post synaptic neurone after activation of NMDA receptor-gated Ca^{2+} conductances (Mayer et al., 1984) and voltage dependent Ca^{2+} channels (Siegelbaum and Kandel, 1991). It is now well established that increases in cAMP and the activation of PKA play a critical role in the induction of long-term synaptic, cellular and behavioural changes implicated in Long term facilitation in *Aplysia* (Bacskai et al., 1993), Drosophila (Davis et al.,

1995), honey bee (Fiala et al., 1999) and mice (Abel et al., 1997), and it has been demonstrated that PKA is specifically involved in the switch from short to long-term memory (Frank and Greenberg, 1994). Despite the differing circumstances in these quite distinct forms of synaptic plasticity, PKA is a key factor in common that appears to play a crucial role in the sensitisation of responsiveness.

During acquisition of the long-term process, serotonin, a facilitating neurotransmitter induces a persistent increase in cAMP dependent protein phosphorylation in sensory neurones (Sweatt and Kandel, 1989) and prolonged application or intracellular injection into the sensory neurone of cAMP produces long-term increases in synaptic strength (Schacher et al., 1988). PKA plays a critical role in the consolidation of long-term memory (Abel et al., 1997), and inhibition of PKA completely blocks the late phase of LTP (L-LTP) in hippocampal CA1 neurones, and activators of PKA facilitate the neurones in a similar manner to LTP (Frey et al., 1993). It has been demonstrated that the amount of regulatory subunits of PKA is decreased when compared with catalytic subunits (Greenberg et al., 1987; Bergold et al., 1990), which results in an imbalance of the R:C ratio and subsequent persistent activation of the kinase. One of the key consequences of PKA activation is the activation of CREB which has been implicated in long-term memory formation in *Aplysia* (Bartsch et al., 1995, 1998 Dash et al., 1990; Alberini et al., 1994) Drosophila (Yin et al., 1994; 1995) and rodents (Silva et al., 1998).

Studies using mice selectively lacking the gene encoding the neuronal specific isoform of the type I regulatory subunit (RIβ) (Brandon et al., 1995) show normal responses in tests of acute nociception (Malmberg et al., 1997). In contrast, PKA RIβ mutant mice show a marked reduction of persistent pain after tissue injury (eg. second phase of the formalin test). The second phase of the formalin test is known to result from both inflammation-evoked primary afferent activity and from sensitisation of dorsal horn neurones (Dickenson and Sullivan, 1990; Puig and Sorkin, 1996). PKA RIβ mice also displayed reduced formalin evoked paw swelling suggesting the reduced pain behaviours may be a result of attenuated peripheral inflammation in the formalin test (Malmberg et al., 1997). However, in contrast to

our findings, PKA RIβ mutant mice also showed normal development of behavioural and anatomical changes in the spinal cord after peripheral nerve injury. This lack of effect is difficult to interpret since RIβ is only one of several similar isoforms; secondly, the unconditional knockout may cause adaptive changes in other subunits or invoke alternative mechanisms and finally, the major role of PKA may be fulfilled by a constitutitively-active form of the enzyme, de-regulated by degeneration of the regulatory subunits.

In summary, inhibitors of activated PKA can selectively inhibit neuropathic sensitisation, which is believed to underlie the development of neuropathic sensitisation following CCI. Expression of the catalytic subunits of PKA is increased in the spinal dorsal horn ipsilateral to nerve injury, which taken together with the decrease in the neurospecific type-I regulatory subunit of PKA (RIβ) suggests that CCI leads to an imbalance in the R:C ratio and persistent activation of PKA catalytic activity. The decrease in RIβ protein levels observed following CCI, provides further evidence of the importance of RIβ in nociceptive processing. These novel findings when considered with the substantial evidence implicating PKA in long-term changes associated with memory formation suggest that persistent activation PKA following CCI may be important for the development of the increased neuronal excitability and persistent nociceptive processing. Parallel studies examining LTF in *Aplysia* have demonstrated that the degradation of PKA regulatory subunits observed in the switch from short to long term memory storage is due to targeted degradation by the ubiquitin proteasome system. The role of the ubiquitin proteasome system in nociceptive processing following CCI is addressed in chapter 5.

CHAPTER 5: THE ROLE OF THE UBIQUITIN-PROTEASOME SYSTEM IN NEUROPATHIC PAIN

5.1 The Ubiquitin-Proteasome System

The ubiquitin-proteasome system is involved in various cellular functions including degradation of obsolete abnormal or damaged proteins (Hershko and Ciechanover, 1982), regulation of the cell cycle by degradation of cyclin dependent kinases (Hershko et al., 1994), and regulation of signal transduction by the degradation of pathway components, for example the regulatory subunits of PKA (Chain et al., 1999). Intracellular protein degradation is a highly selective process. Some proteins are degraded within minutes, while others are practically stable (Coux et al., 1996). Usually, regulatory proteins or enzymes have fast turnover rates so that their levels can be rapidly changed in response to appropriate stimuli. In some cases, the rates of degradation of regulatory proteins are controlled with high precision. In the ubiquitin-proteasome system, proteins destined for degradation are ligated to the polypeptide ubiquitin, and then are degraded by a specific protease complex that acts on ubiquitinated proteins.

Ubiquitination is a three-step process. First, ubiquitin is activated by a ubiquitin activating enzyme (E1). E1 is the first enzyme in the ubiquitination process. In the presence of ATP and magnesium E1 forms a high-energy thiol ester bond with ubiquitin (Haas et al., 1982; Hershko and Ciechanover, 1992). The activated ubiquitin is then transferred to a ubiquitin carrier protein (E2). E2 proteins are a family of enzymes which shuttle ubiquitin between E1 and protein substrates or E3 proteins. More than a dozen E2 enzymes have been identified (Jentsch, 1992). Different E2 enzymes may have different functions and different substrate specificity (Pickart and Rose, 1985; Pickart and Vella, 1988; Hershko and Ciechanover, 1992; Jentsch, 1992; Wing et al., 1992). Finally, ubiquitin is conjugated to a protein substrate by forming an isopeptide bond between the C-terminal glycine residue of ubiquitin and the amino terminal lysine residue of the protein substrate (Gregori et al., 1990). This conjugation step requires a ubiquitin protein ligase enzyme (E3).

Multiple molecules of ubiquitin can be ligated to a protein substrate to form poly-ubiquitin chains (Chau et al., 1989). Polyubiquitinated proteins are targets for degradation by the proteasome, a large multi subunit particle found in the nucleus and cytoplasm of eukaryote cells known as the 26S proteasome. The 26S proteasome is composed of a 20S catalytic core and a 19S regulatory complex (Coux et al., 1996).

5.2 Ubiquitin Carboxyl-Terminal Hydrolases (UCHs)

Essential to the operation of the ubiquitin system is the recycling of free ubiquitin. Ubiquitin carboxyl-terminal hydrolase (UCH) is required to release ubiquitin from linkage with the protein substrate (Figure 5.1). UCHs are a class of small cytoplasmic thiol proteases with specificity for cleavage of small esters and amides of the carboxyl-terminal glycine of ubiquitin. In addition, UCH isopeptidase activity may be expected to disassemble polyubiquitin chains linked to the protein substrate, following the degradation of the protein. Several UCHs have been characterised: UCH-L1 is among the most abundant proteins of brain, constituting from 1 to 5 % of the soluble protein. It is a cytoplasmic protein of 212 amino acids (Day et al., 1989); a neuronal-specific protein strongly expressed in neuronal, neuroendocrine, and perhaps some fetal cells. Isozyme L3 is present mainly in haemopoietic cells and many tissues and cells contain isozyme L2, which appears to be a "housekeeping" isozyme (Wilkinson et al., 1992). The expression of UCHs appear to be highly tissue specific, suggesting the role of ubiquitination may vary with tissue type or differentiation.

Figure 5.1 The Ubiquitin-Proteasome System

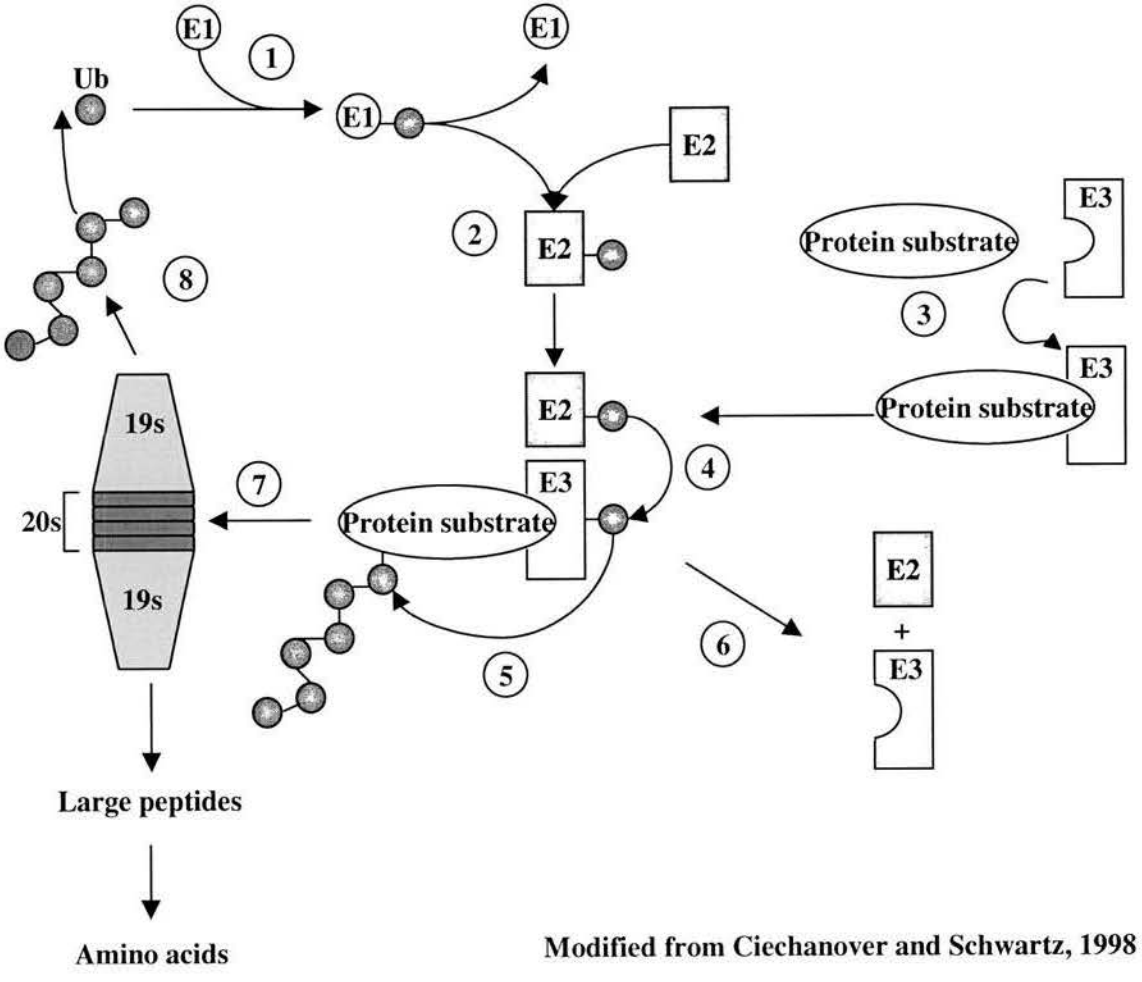
Conjugation of ubiquitin to the target protein

1) Activation of ubiquitin by E1.
2) Transfer of the activated ubiquitin moiety to a member of the E2 family of enzymes.
3) Formation of a binary complex between E3 and the protein substrate.
4) Formation of a ternary E2-E3-protein substrate complex and transfer of the activated ubiquitin moiety from E2 to E3.
5) Synthesis of protein substrate-anchored polyubiquitin chain.
6) Recycling of E2 and E3.

Degradation of the polyubiquitin-conjugated substrate by the proteasome

7) Transfer of the protein substrate-ubiquitin adduct to the ubiquitin proteasome, and subsequent proteolysis of the protein substrate into constituent amino acids.
8) Recycling of ubiquitin by ubiquitin C-terminal hydrolases (isopeptidases).

The Ubiquitin Proteasome System



Modified from Ciechanover and Schwartz, 1998

5.3 The Ubiquitin-Proteasome System in Sensory Transmission

The results in chapter 4 highlighted a potential role for PKA in neuropathic pain following peripheral nerve injury. Activation of PKA also plays an important role in producing long term facilitation (LTF) in *Aplysia* sensory neurones. In the LTF model, pre-synaptic facilitation of sensory to motor synapses is thought to underlie behavioural sensitisation, a modification of defensive reflexes that is considered a simple form of memory (Byrne and Kandel, 1996; Greenberg et al., 1987). Sustained sensory neurone activation induces the dissociation of PKA regulatory subunits and the translocation of the constitutively active free catalytic subunits to the cell nucleus. A stable change in the R:C ratio is believed to account for the long lasting persistent activation of the kinase seen in LTF, and implies the existence of an important mechanism for regulating the ratio of PKA subunits.

In *Aplysia* sensory neurones there is increasing evidence suggesting that the ubiquitin-proteasome system plays a vital role in the regulation of PKA activity by a mechanism involving generation of persistently active autonomous PKA (Chain et al., 1999; Hegde et al., 1997). The ubiquitin-proteasome system is a major non-lysosomal proteolytic pathway that degrades diverse cellular proteins, including a number of proteins with important roles in the regulation of cell growth or function. The functional activity of the proteasome is enhanced by the UCH family of isopeptidases (for example UCH-L1), which appear to play a crucial role in maintaining activity by preventing accumulation of inhibitory polyubiquitin chains (Wilkinson, 1997). Hegde et al., (1997) demonstrated that the induction of UCH in *Aplysia* is important for LTF and the hydrolase enhances proteasome-mediated substrate degradation in vitro. The ubiquitin-proteasome system appears to regulate PKA activity by degrading its regulatory subunits, which normally maintain PKA in an inactive state. Stimulus-induced degradation of PKA regulatory subunits is thus thought to lead to persistent activation of the kinase and maintenance of the synapse in a facilitated state until cAMP is provided (Greenberg et al., 1987; Chain et al., 1999). Therefore, protein degradation by this pathway is considered to be important for the control of PKA activity observed in the switch from short to long term facilitation and the establishment of a persistently active PKA (Hegde et al., 1993).

5.4 Aims of the Present Experiments

Given the role of targeted proteolysis of the R subunits of PKA in LTF in *Aplysia*, and the similarities between the role of PKA in LTF and central sensitisation, experiments sought to identify the possible contribution made by the ubiquitin-proteasome system to the persistent activation of PKA observed in central sensitisation following peripheral nerve injury and in the maintenance of abnormal behaviours following CCI. The effects of selective inhibitors of the ubiquitin-proteasome system were examined on the sustained firing of dorsal horn neurones and on reflex withdrawal responses. The levels of the rat homologue of *Aplysia* UCH (UCH-L1) were also examined at both the mRNA level and protein level.

10-14 days following CCI surgery, electrophysiological techniques were employed to determine the role of the ubiquitin-proteasome system in the transmission of sensory information. Extracellular recordings were made from individual spinal dorsal horn neurones while inhibitors of the proteasome (MG-132 and lactacystin) were ionophoretically applied nearby. Neuronal activity was induced by (i) light brushing of the cutaneous receptive field, (ii) cold stimulation via a thermal Peltier probe or (iii) topical application of the chemical algogen mustard oil.

To assess the contribution of the ubiquitin-proteasome system in the maintenance of thermal hyperalgesia, mechanical and cold allodynia following CCI the effects of spinally administered proteasome inhibitors were examined. In conscious rats, inhibitors of the ubiquitin-proteasome system (MG-132 and epoxomicin) were administered intrathecally 10-14 days following CCI surgery (at the peak of behavioural changes) and reflex withdrawal responses to heat, mechanical and cold stimuli were recorded. Responses were also obtained from normal rats for comparison.

To assess any alterations in the levels of expression of UCH-L1 in the lumbar dorsal horn following CCI, in situ hybridisation histochemistry and Western blot analysis of the expression of mRNA and protein for UCH-L1 were carried out. Levels of UCH-

L1 mRNA and protein were also measured in the lumbar dorsal horn of CCI rats and normal and sham-operated rats. The methods are described in detail in chapter 2.

5.5 Results

5.5.1 Sensory Responses of Single Dorsal Horn Neurones: Application of Inhibitors of the Proteasome Complex

These results are based on data obtained from a total of 35 neurones in 35 rats,(n=13 CCI, n=15 normal and n=7 sham-operated). Extracellular recordings were made from single dorsal horn neurones situated in lamina III-V. Microelectrode depths ranged from 200-1000μm from the surface of the spinal cord, which was measured using electrode contact at the dorsal surface and dye spot deposition in histological sections following experimentation.

Multireceptive neurones were studied in this investigation. To facilitate neuronal classification and to obtain sustained neuronal activation following mustard oil application, only neurones with receptive fields on the hairy skin of the hind limb were investigated, corresponding to the area innervated by the constricted part of the sciatic nerve (Schmalbruch, 1986).

5.5.1.1 Effects of Ubiquitin-Proteasome Inhibitors on Sustained Neuronal Firing Induced by Brush, Cold or Mustard Oil Application

Neuronal firing was induced by stimulating the cutaneous receptive field using (i) a motorised rotating brush, (ii) a thermally maintained Peltier device or (iii) topical application of mustard oil. Generally 1 min of activity was recorded before ionophoresis of inhibitors commenced. Neuronal activation to either application of innocuous brush or mustard oil stimuli to the peripheral receptive field was found in the majority of neurones recorded. However, activation of neurones to cold stimuli (4°C) was only found in neuropathic animals, which is in agreement with the observation that it is only neuropathic animals that develop a paw withdrawal response to cold (Simone and Kajander, 1996). The spontaneous firing rates of neurones were low (1-2Hz) and those in response to the stimuli were between 12 and 50Hz. For each neurone, in the majority of cases, the rates of firing in response to

the different stimuli, prior to drug application, was within 15Hz. No significant change in activity was ever observed during control ejection of saline (up to 60nA for 5 min).

5.5.1.2 Brush-Induced Activity

In normal animals, ionophoretic application of the proteasome inhibitors lactacystin and MG-132 showed negligible effects when applied to the sustained brush-induced activity of dorsal horn neurones. However, application of lactacystin and MG-132 significantly inhibited brush-induced activity of dorsal horn neurones, ipsilateral to the nerve injury in CCI animals (Figure 5.2).

5.5.1.3 Cold-Induced Activity

Over half of the dorsal horn neurones tested responded strongly to a cold stimulus. There was some variability in the firing responses of the dorsal horn neurones tested, with some neurones being more susceptible to the decreasing temperature while others had a greater firing activity during the sustained cold response. However, all neurones used produced consistent responses following each cold stimulus, giving reproducible peaks of activity of 5-28Hz. The proteasome inhibitors lactacystin and MG-132 showed significant inhibitory effects when applied during the repeated cold-induced activity (Figure 5.3).

5.5.1.4 Mustard Oil-Induced Activity

In normal animals, ionophoretic application of the proteasome inhibitors lactacystin and MG-132 caused significant inhibition of mustard oil-induced activity of dorsal horn neurones. However, application of lactacystin and MG-132 inhibited mustard oil-induced activity to a consistently greater extent in CCI animals (Figure 5.4).

Table 5.1 is a summary table of the results indicating the overall effects of lactacystin and MG-132 on stimulus-evoked activity of dorsal horn neurones in normal, sham-operated and neuropathic animals with analysis of the statistical significance of effects.

Figure 5.2 Effects of Ionophoretic Application of the Proteasome Inhibitors Lactacystin and MG-132 on Sustained Brush-Induced Responses of Dorsal Horn Neurones in Normal and CCI Rats

Records of ongoing firing of individual neurones are displayed as number of action potentials per second (A/s), integrated over 1000msec bins, plotted against time.

Traces (A) and (B) show the observed lack of effect of ionophoretic application of selective proteasome inhibitors lactacystin and MG-132 on the sustained neuronal firing induced by a motorised rotating brush in normal animals. Traces (C) and (D) show the inhibitory effect of ionophoretic application of lactacystin and MG-132 on brush-evoked activity in neuropathic animals. These results are typical for the neurones tested in this study.

Drugs were ejected ionophoretically at 10nA increments from a minimum of 10nA up to a maximum of 60nA if required.

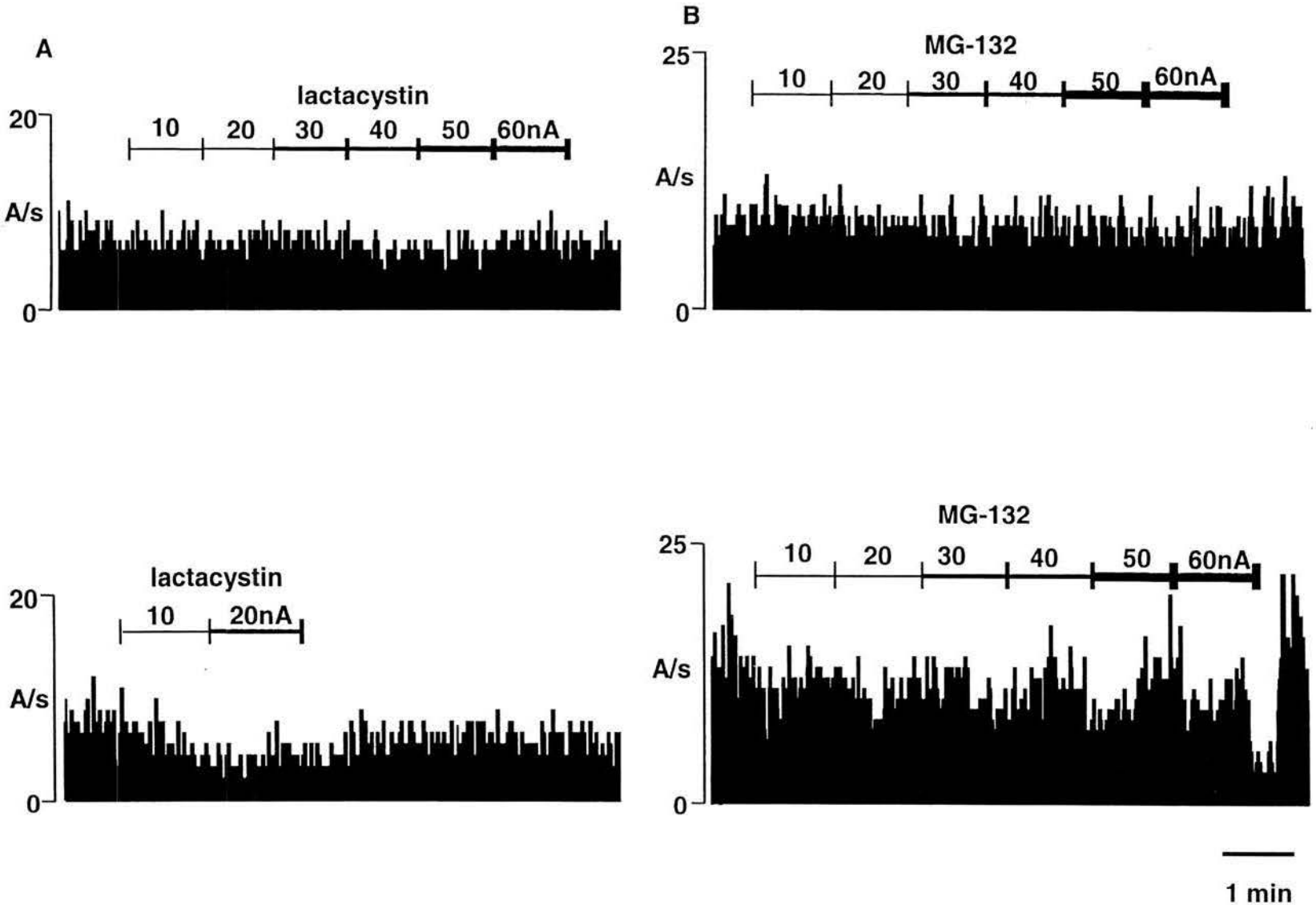

A
lactacystin
10
20
30
40
50
60nA
20
A/s
0
B
MG-132
10
20
30
40
50
60nA
25
A/s
0
lactacystin
10
20nA
20
A/s
0
MG-132
10
20
30
40
50
60nA
25
A/s
0
1 min

Figure 5.3 Effects of Ionophoretic Application of the Proteasome Inhibitors Lactacystin and MG-132 on Cold-Induced Firing of Dorsal Horn Neurones in CCI Rats

Records of ongoing firing of individual neurones are displayed as number of action potentials per second (A/s), integrated over 1000msec bins, plotted against time.

Traces (A) and (B) show the typical inhibitory effects of ionophoretic application of selective proteasome inhibitors lactacystin and MG-132 on 10s pulses of neuronal activity induced by repeated stimulation of the cutaneous receptive field by a cold Peltier device at 5°C (■). The X-axis breaks represent the 5 min recovery period required for neuronal firing rates to return to pre-drug control levels. These results are typical for the population of cells tested in this study. Intense responses to the cold stimulus were absent from non-neuropathic animals.

Drugs were ejected ionophoretically at 10nA increments from a minimum of 10nA up to a maximum of 60nA if required.

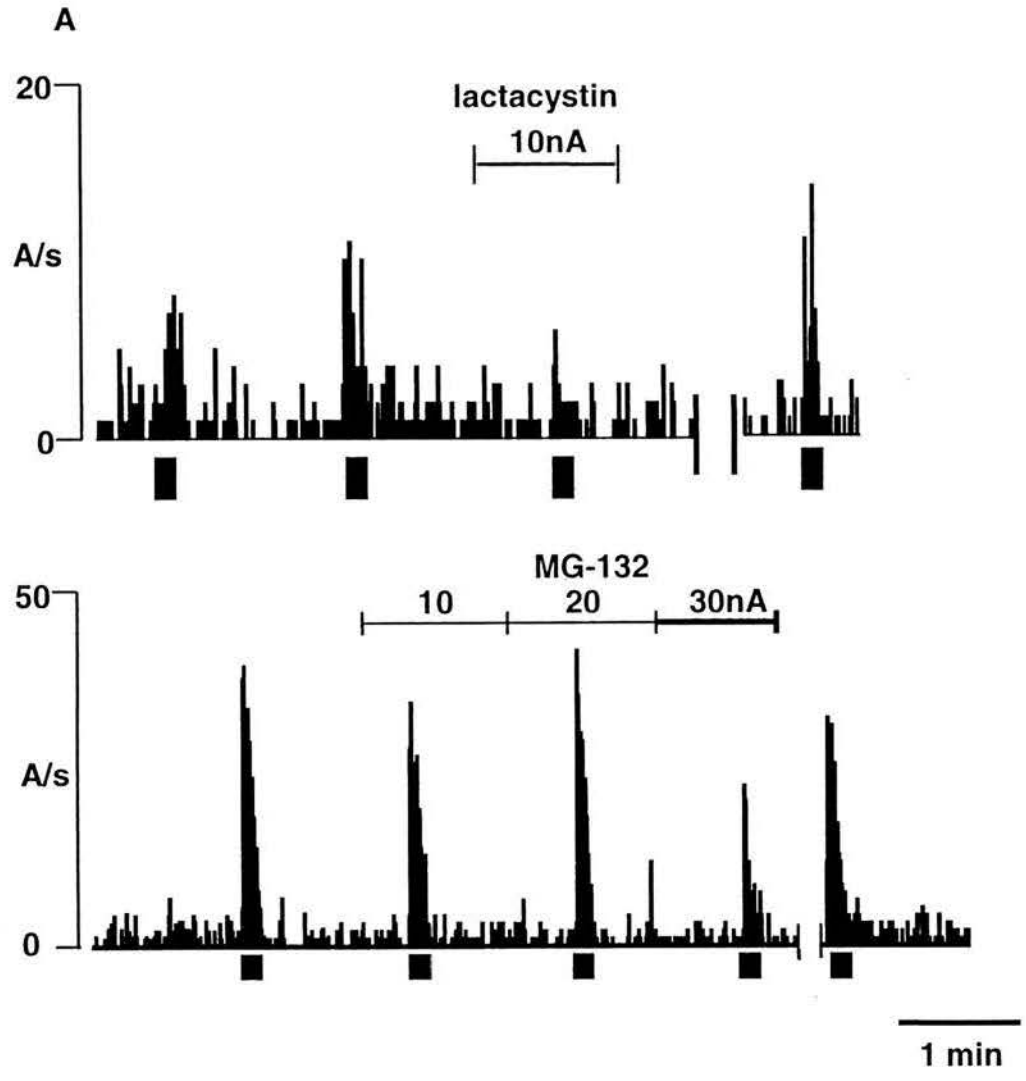

A
20
lactacystin
10nA
A/s
0
MG-132
50
10
20
30nA
A/s
0
1 min

Figure 5.4 Effects of Ionophoretic Application of the Proteasome Inhibitors Lactacystin and MG-132 on Sustained Mustard Oil-Induced Activity of Dorsal Horn Neurones in Normal and CCI Rats

Records of ongoing firing of individual neurones are displayed as number of action potentials per second (A/s), integrated over 1000msec bins, plotted against time.

Traces (A) and (B) represent the significant inhibitory effects of ionophoretic application of selective proteasome inhibitors lactacystin and MG-132 respectively on the sustained mustard oil-induced neuronal firing in normal animals. Traces (C) and (D) represent CCI animals and showed an apparently increased inhibition of sustained mustard oil-induced activity following ionophoretic application of lactacystin and MG-132 respectively. Each trace is from a different neurone and is typical of the results obtained from these experiments.

Drugs were ejected ionophoretically at 10nA increments from a minimum of 10nA up to a maximum of 60nA if required.

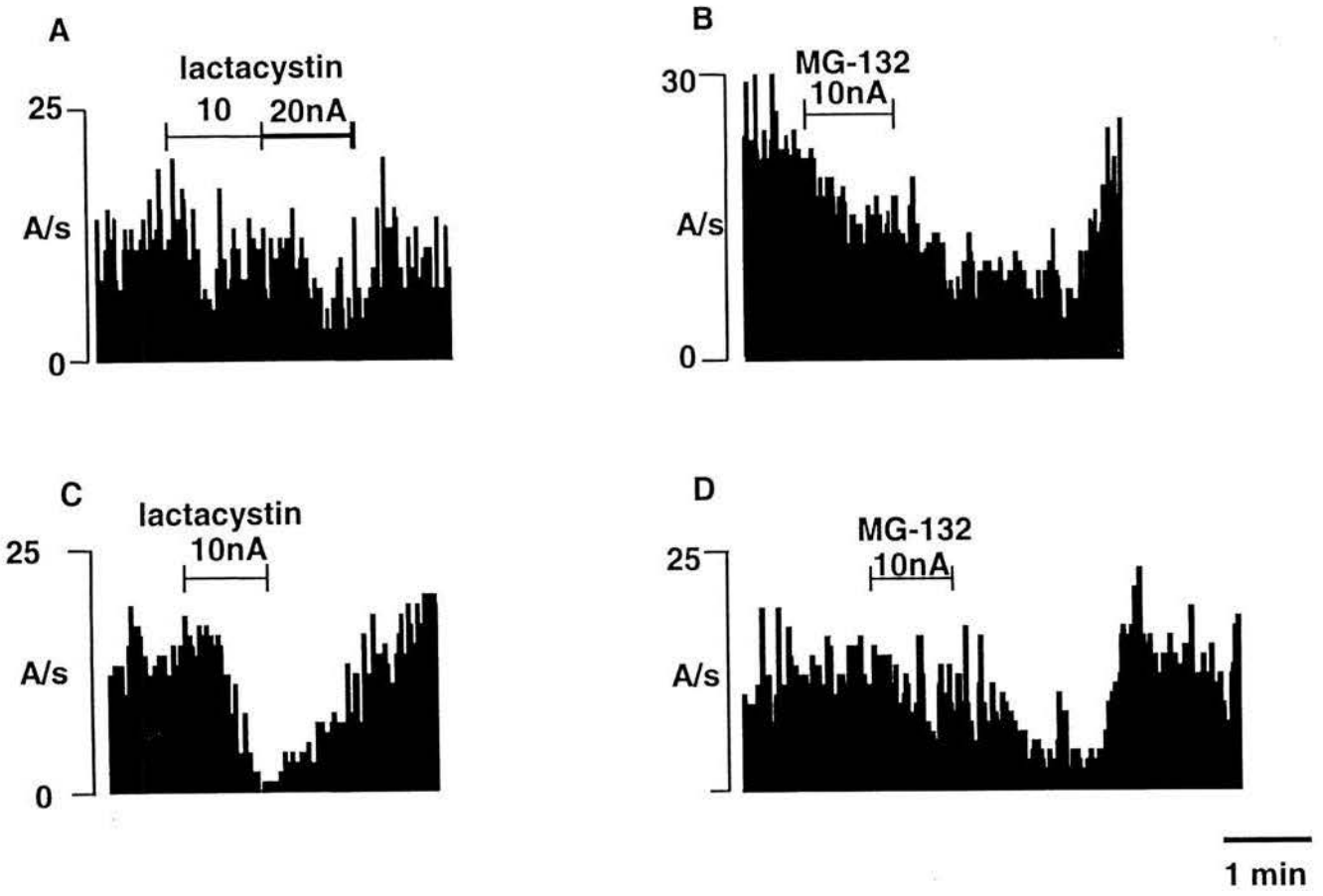

A
lactacystin
10 20nA
25
A/s
0
B
MG-132
10nA
30
A/s
0
C
lactacystin
10nA
25
A/s
0
D
MG-132
10nA
25
A/s
1 min

Table 5.1 Effects of Ionophoretically Applied Proteasome Inhibitors on Sensory Responses of Single Dorsal Horn Neurones in Normal and CCI Rats

Summarised data representing the effects of proteasome inhibitors lactacystin and MG-132 on single dorsal horn neurone sensory responses. Data is expressed as mean ± SEM together with number of cells recorded. The range of ionophoretic currents required to reach a steady state of depression of firing rate are indicated.

Using mean firing rates over 15s time bins, the statistical significance of differences between maximally inhibited firing rates in the presence of drug and firing rates immediately prior to ionophoresis were calculated using Kruskal-Wallis one way ANOVA, followed by Dunn's post-hoc test (* $p \leq 0.05$). The range of ionophoretic currents required to reach a maximal depression of firing rate are indicated. Further increases in currents beyond these values caused no greater inhibition.

Drug	Condition	% of Stimulus-Evoked Response Remaining		
		Brush	Mustard oil	Cold
Lactacystin	Normal	87.5 ± 5.9 (n=8) (10-60nA)	58.6 ± 7.1* (n=7) (10-60nA)	-
	Sham	79.2 ± 1.6 (n=3) (50-60nA)	50.1 ± 6.2* (n=4) (10-40nA)	-
	CCI	60.8 ± 4.2* (n=4) (20-60nA)	34.1 ± 5.1* (n=6) (20-40nA)	58.7 ± 4.7* (n=3) (10-30nA)
MG-132	Normal	76.4 ± 2.5 (n=4) (10-40nA)	57.1 ± 8.8* (n=4) (20-40nA)	-
	CCI	66.4 ± 5.4* (n=4) (30-60nA)	31.6 ± 2.1* (n=7) (10-30nA)	48.3 ± 7.4* (n=3) (10-30nA)

5.5.2 Effects of Intrathecal Administration of the Selective Proteasome Inhibitors MG-132 and Epoxomicin on Sensory Reflex Withdrawal Responses in Normal and CCI Rats

Results were obtained from a total of 47 rats, (n=39 CCI and n=8 normal). Intrathecal administration of the selective proteasome inhibitors MG-132 and epoxomicin was carried out as described previously (chapter 2 section 2.3.4.). In rats exhibiting peak behavioural changes following CCI, both proteasome inhibitors significantly reversed the increased reflex withdrawal responses to noxious heat, innocuous mechanical stimulation and innocuous cold that are indicative of thermal hyperalgesia, mechanical and cold allodynia respectively (Figure 5.5, 5.6 and 5.7). This inhibition was monitored over a 90 minute testing period. Control intrathecal administration of vehicle in CCI rats had no significant effect on reflex responses in all three sensory tests (Figure 5.5C, 5.6C and 5.7C) and administration of the proteasome inhibitors in normal animals also had no significant effect (Figure 5.8, 5.9). Intrathecal administration of the selective NF-κB inhibitor parthelonide was carried out as a pilot investigation to assess the possibility of NF-κB involvement since the ubiquitin-proteasomal system can degrade the inhibitory partner I-κB and lead to NF-κB (Figure 5.10).

Figure 5.5 Effects of Intrathecal Administration of the Proteasome Inhibitors Epoxomicin, MG-132 and Vehicle on Reflex Withdrawal Responses to Noxious Heat in CCI Rats

Data are represented as mean paw withdrawal latency (s) for ipsilateral and contralateral paws plotted against time (min) pre and post-injection. Arrow marks intrathecal drug administration. In rats exhibiting peak behavioural changes following CCI, paw withdrawal latency to noxious heat ipsilateral (but not contralateral) to nerve injury showed significant differences between pre- and post-drug administration values († $p \leq 0.05$; one-way ANOVA followed by Neuman-Keuls post-hoc test). Significant differences between contralateral and ipsilateral paw withdrawal latency are indicated (* $p \leq 0.05$; Student's paired t-test). (A) Effects of intrathecal administration of epoxomicin [0.75nmol in 50µl], (n=8). (B) Effects of intrathecal administration of MG-132 [5nmol in 50µl], (n=8). (C) Effects of intrathecal administration of vehicle (50µl of 0.5% dimethylformamide in saline, n=4).

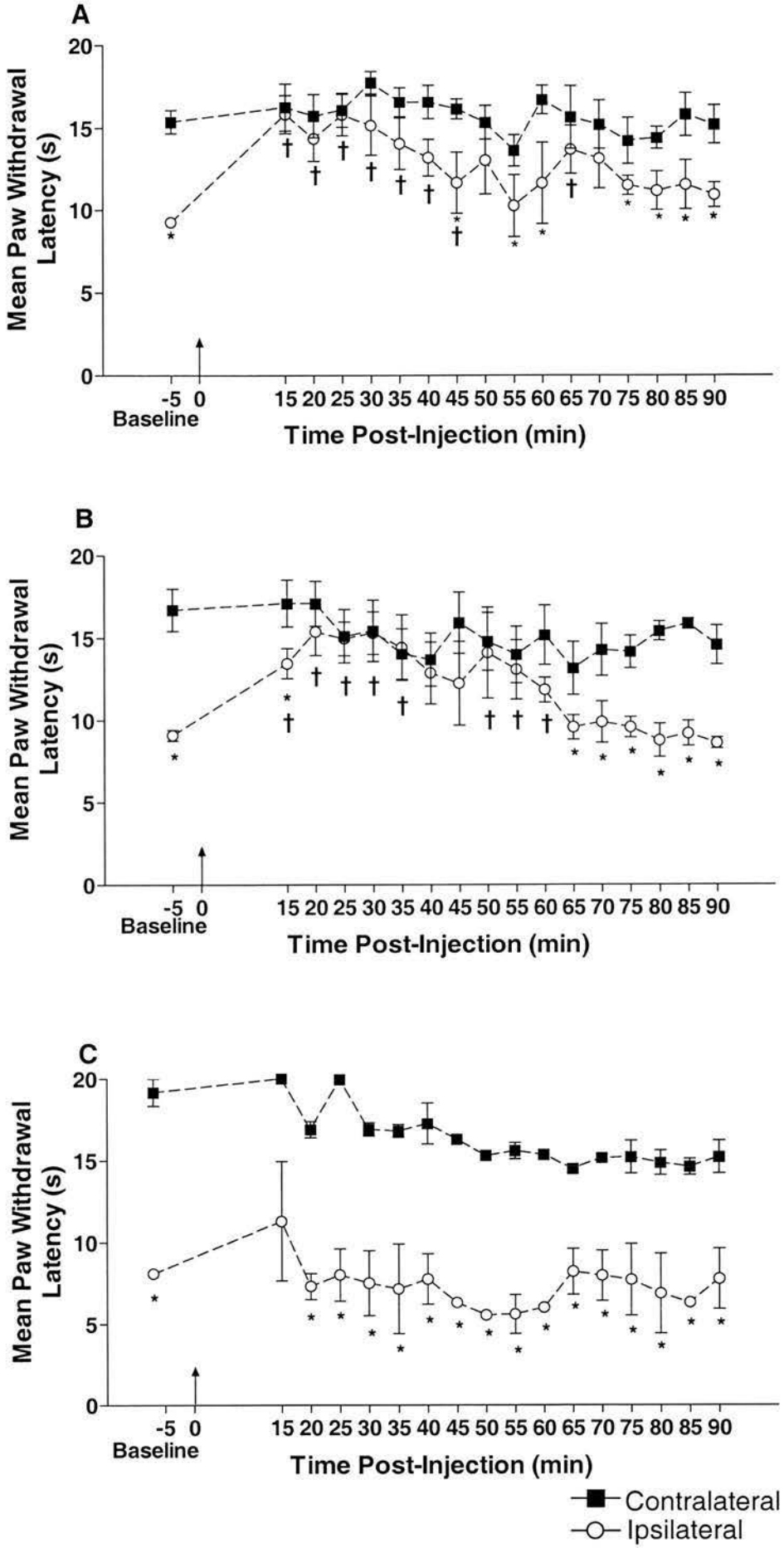

A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
B
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
C
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
Contralateral
Ipsilateral

Figure 5.6 Effects of Intrathecal Administration of the Proteasome Inhibitors Epoxomicin, MG-132 and Vehicle on Reflex Withdrawal Responses to Innocuous Mechanical Stimuli in CCI Rats

Data are represented as mean paw withdrawal threshold (mN/mm^2) for ipsilateral and contralateral paws plotted against time (min) pre and post-injection. Arrow marks intrathecal drug administration. In rats exhibiting peak behavioural changes following CCI, paw withdrawal threshold to innocuous mechanical stimulation ipsilateral (but not contralateral) to nerve injury showed significant differences between pre- and post-drug administration values († $p \leq 0.05$; Kruskal-Wallis ANOVA followed by Dunn's post-hoc test). Significant differences between contralateral and ipsilateral paw withdrawal thresholds are indicated (* $p \leq 0.05$; Mann-Whitney U test). (A) Effects of intrathecal administration of epoxomicin [0.75nmol in 50µl], (n=8). (B) Effects of intrathecal administration of MG-132 [5nmol in 50µl], (n=8). (C) Effects of intrathecal administration of vehicle (50µl of 0.5% dimethylformamide in saline, n=4).

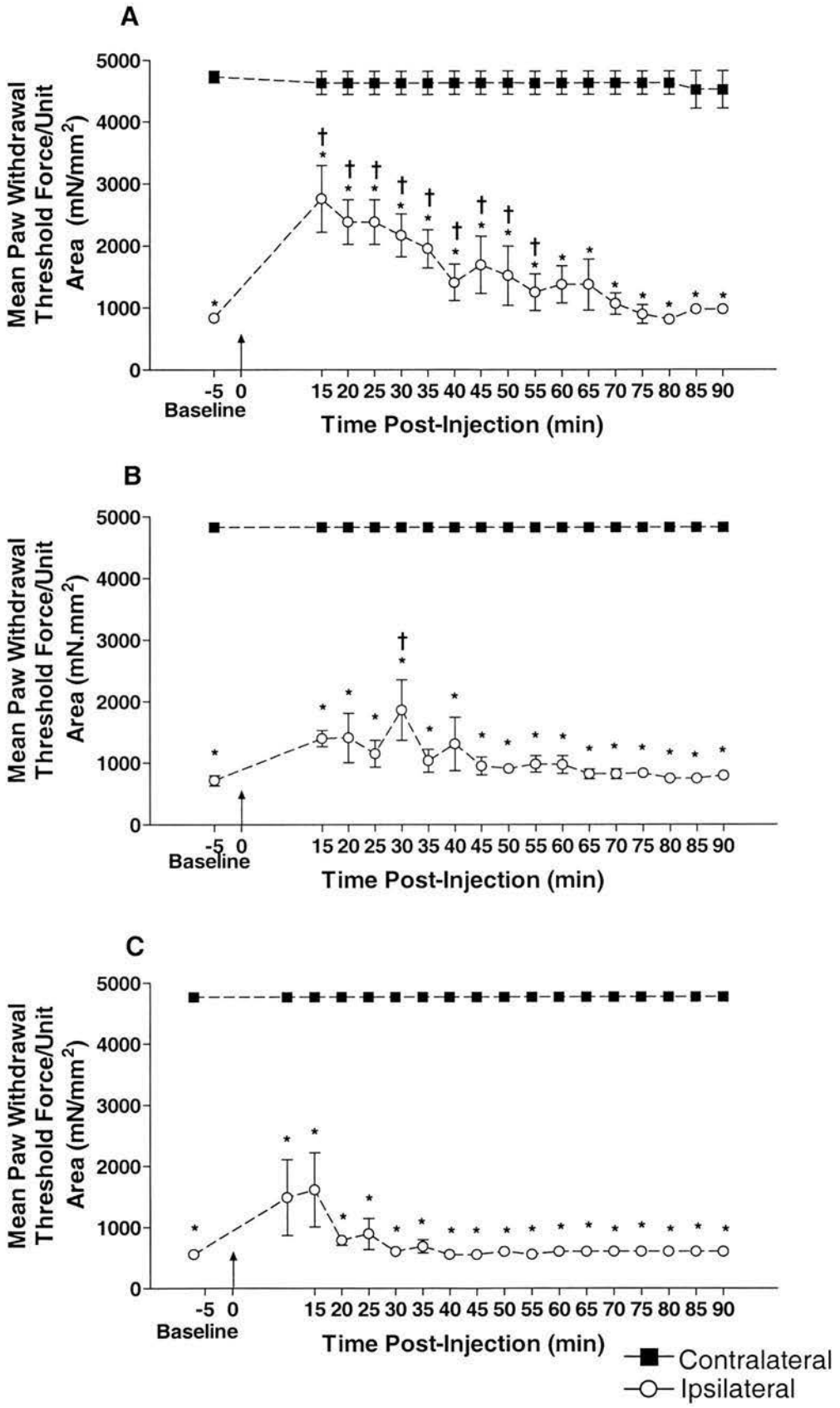

A
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm²)
5000
4000
3000
2000
1000
0
-5 0
Baseline
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN.mm²)
5000
4000
3000
2000
1000
0
-5 0
Baseline
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
C
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm²)
5000
4000
3000
2000
1000
0
-5 0
Baseline
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
Contralateral
Ipsilateral

Figure 5.7 Effects of Intrathecal Administration of the Proteasome Inhibitors Epoxomicin, MG-132 and Vehicle on Reflex Withdrawal Responses to Innocuous Cold Stimuli in CCI Rats

Data are represented as mean suspended paw elevation time (SPET (s)) for ipsilateral and contralateral paws plotted against time (min) pre and post-injection. Arrow marks intrathecal drug administration. In rats exhibiting peak behavioural changes following CCI, suspended paw elevation time ipsilateral (but not contralateral) to nerve injury showed significant differences between pre- and post-drug administration values († $p \leq 0.05$; one-way ANOVA followed by Neuman-Keuls post-hoc test). Significant differences between contralateral and ipsilateral paw elevation time are indicated (* $p \leq 0.05$; Student's paired t-test). (A) Effects of intrathecal administration of epoxomicin [0.75nmol in 50µl], (n=8). (B) Effects of intrathecal administration of MG-132 [5nmol in 50µl], (n=8). (C) Effects of intrathecal administration of vehicle (50µl of 0.5% dimethylformamide in saline, n=4).

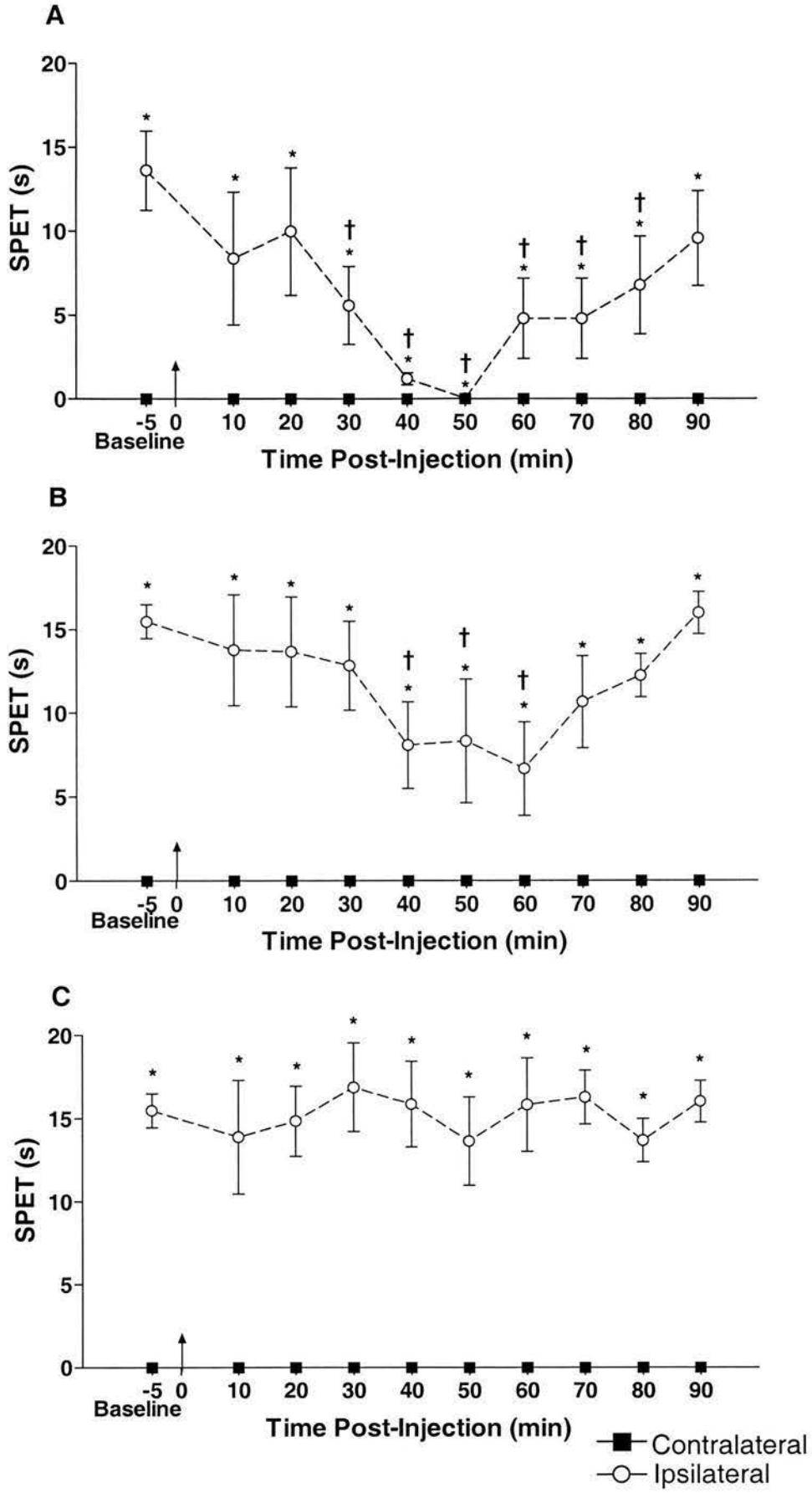

A
SPET (s)
Baseline
Time Post-Injection (min)
B
SPET (s)
Baseline
Time Post-Injection (min)
C
SPET (s)
Baseline
Time Post-Injection (min)
Contralateral
Ipsilateral

Figure 5.8 Effects of Intrathecal Administration of the Proteasome Inhibitor Epoxomicin on Reflex Withdrawal Responses to Noxious Heat and Innocuous Mechanical Stimuli in Normal Rats

Data are represented as mean paw withdrawal latency (s) from noxious heat (A), and mean paw withdrawal threshold (mN/mm^2) to innocuous mechanical stimuli (B) for ipsilateral and contralateral paws plotted against time (min) pre and post-injection. Arrow marks intrathecal drug administration.

In normal unoperated rats, paw withdrawal latency to noxious heat and paw withdrawal threshold to innocuous mechanical stimulation was unaltered following intrathecal administration of epoxomicin. Epoxomicin was administered at a dose of [0.75nmol in 50μl], (n=4).

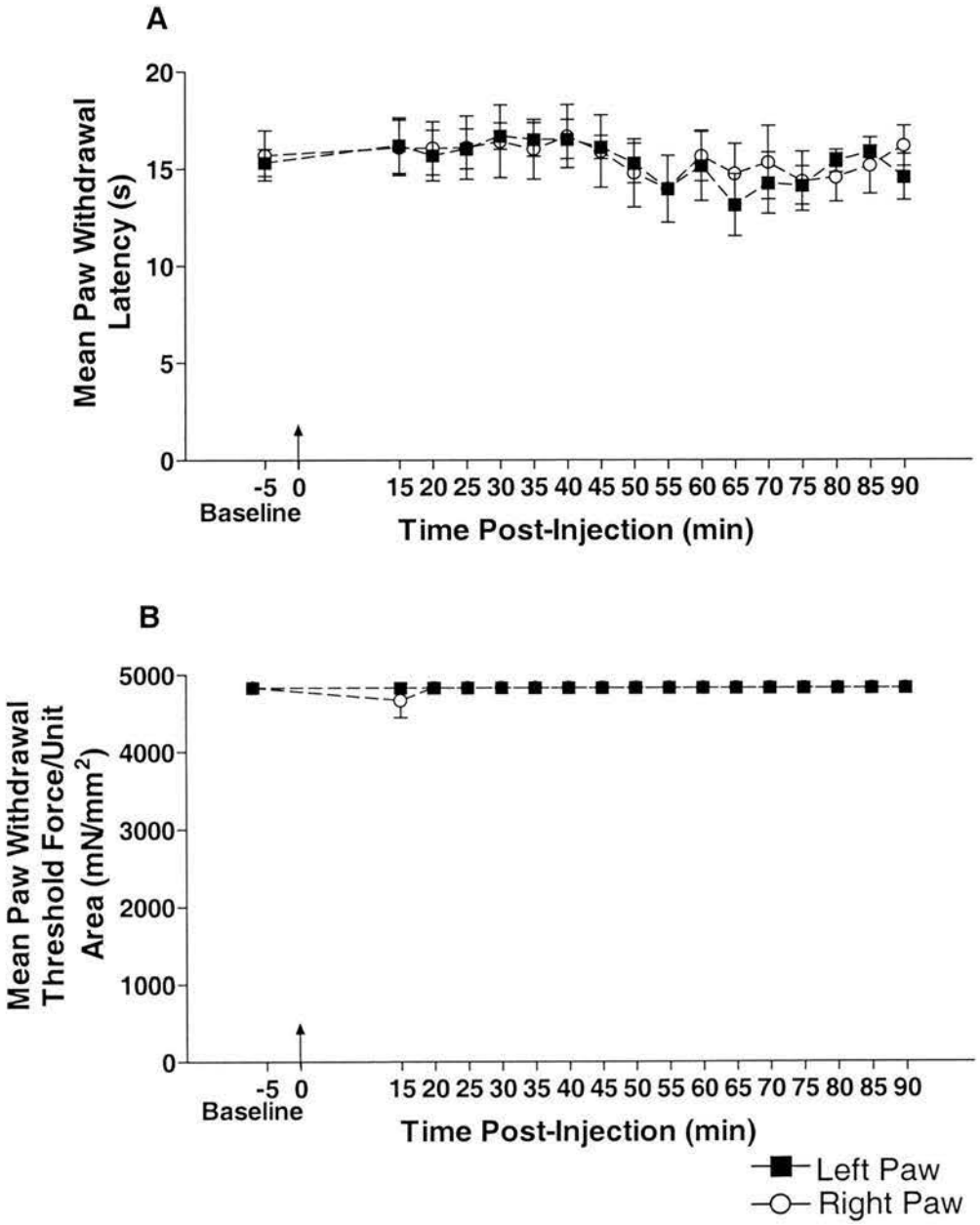

A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm2)
5000
4000
3000
2000
1000
0
-5 0
Baseline
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
Left Paw
Right Paw

Figure 5.9 Effects of Intrathecal Administration of the Proteasome Inhibitor MG-132 on Reflex Withdrawal Responses to Noxious Heat and Innocuous Mechanical Stimuli in Normal Rats

Data are represented as mean paw withdrawal latency (s) from noxious heat (A), and mean paw withdrawal threshold (mN/mm^2) to innocuous mechanical stimuli (B) for ipsilateral and contralateral paws plotted against time (min) pre and post-injection. Arrow marks intrathecal drug administration.

In normal unoperated rats, paw withdrawal latency to noxious heat and paw withdrawal threshold to innocuous mechanical stimulation was unaltered following intrathecal administration of MG-132. MG-132 was administered at a dose of [5nmol in 50µl], (n=4).

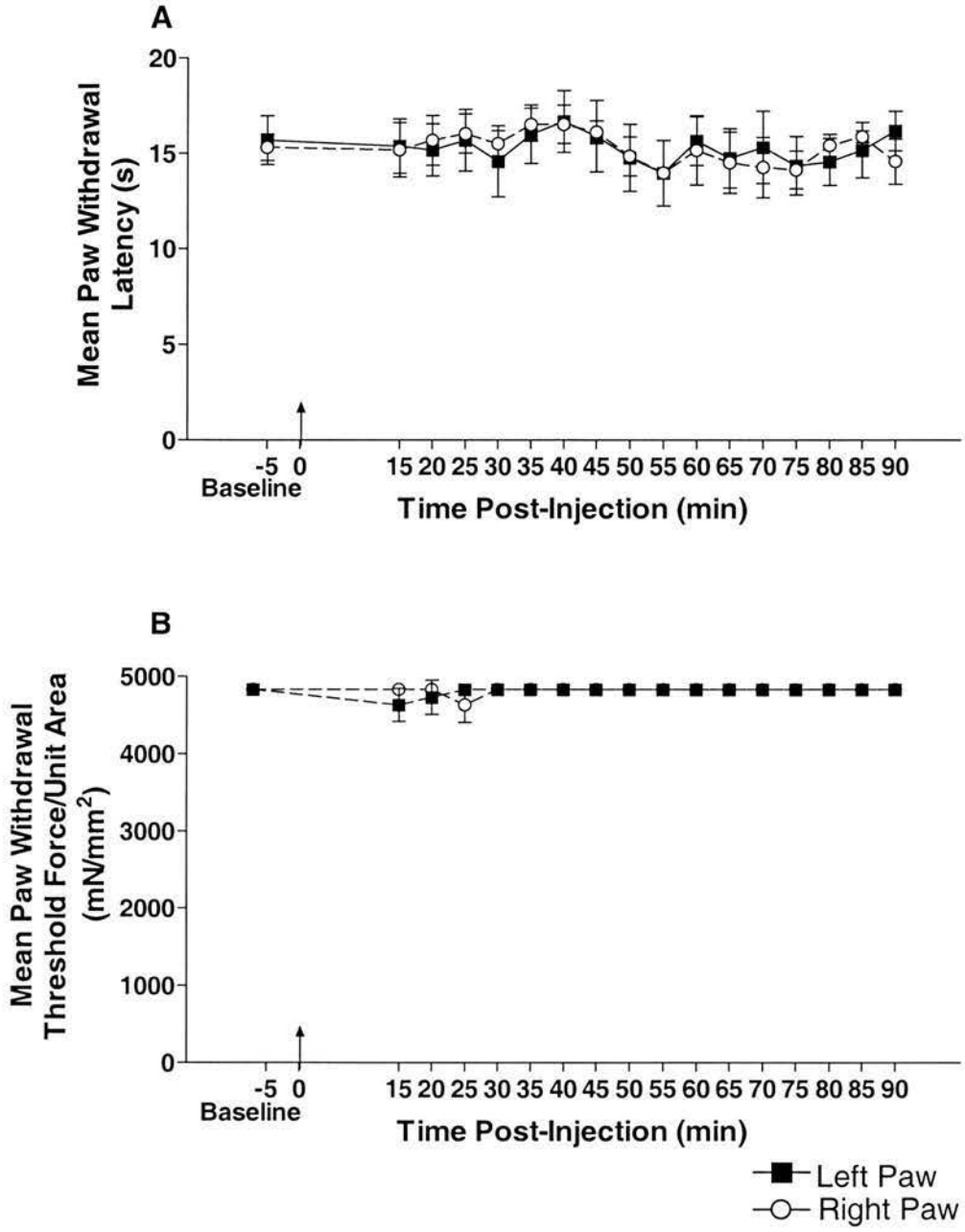
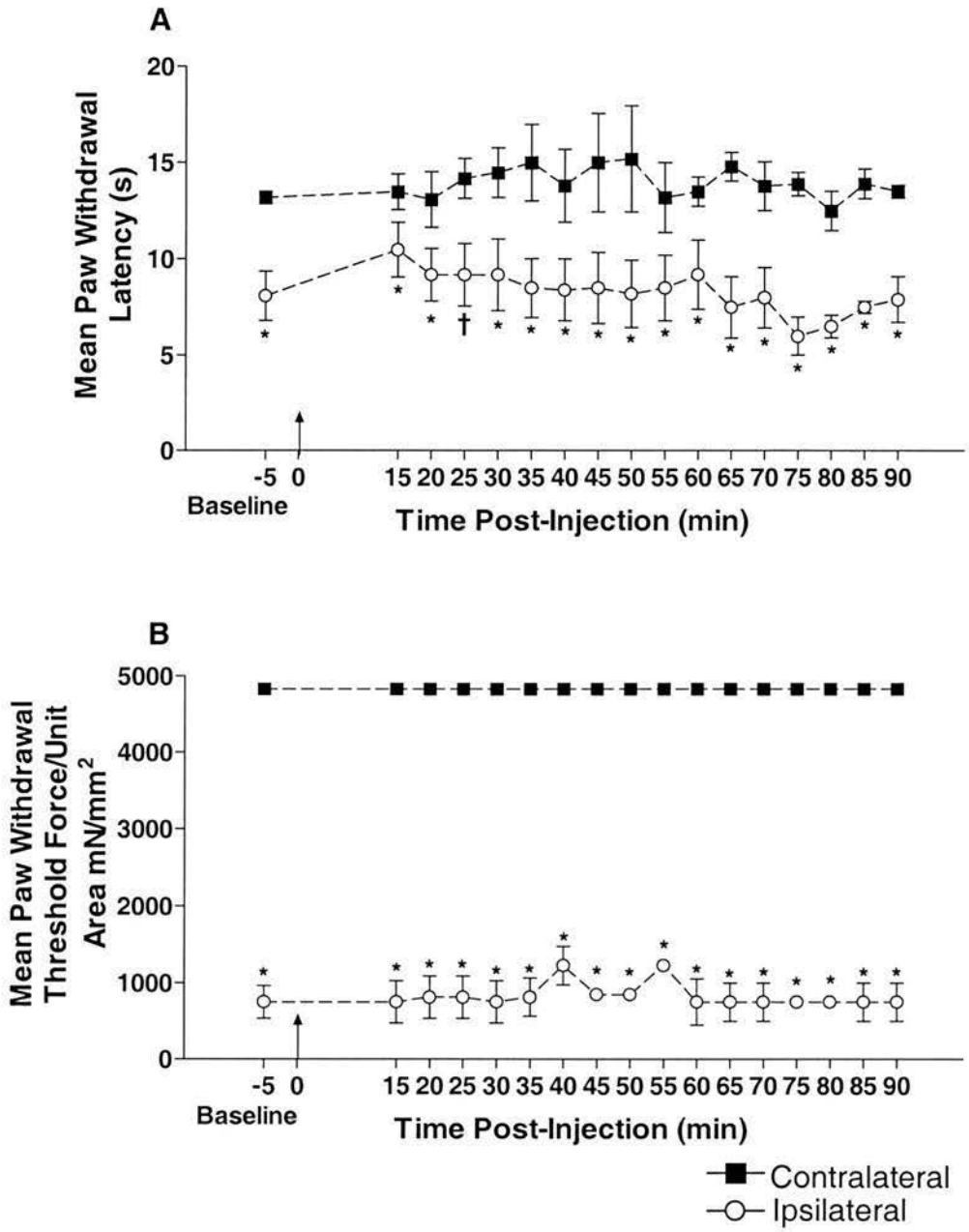

A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area (mN/mm2)
5000
4000
3000
2000
1000
0
-5 0
Baseline
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
Left Paw
Right Paw

Figure 5.10 Effects of Intrathecal Administration of the NF-κB Inhibitor Parthelonide on Reflex Withdrawal Responses to Noxious Heat and Innocuous Mechanical Stimuli in CCI Rats

Data are represented as mean paw withdrawal latency (s) to noxious heat (A), and mean paw withdrawal threshold (mN/mm^2) to innocuous mechanical stimuli (B) for ipsilateral and contralateral paws plotted against time (min) pre and post-injection. Arrow marks intrathecal drug administration.

(A) In rats exhibiting peak behavioural changes following CCI, paw withdrawal latency to noxious heat ipsilateral (or contralateral) to nerve injury showed no significant differences between pre- and post-drug administration values (one way ANOVA). Significant differences between contralateral and ipsilateral paw withdrawal latency and paw withdrawal latencies are indicated (* $p \leq 0.05$; Student's paired t-test). Parthelonide was administered at a dose of [1.5nmol in 50μl], (n=3).

(B) In rats exhibiting peak behavioural changes following CCI, paw withdrawal threshold to innocuous mechanical stimuli ipsilateral (or contralateral) showed no significant differences between pre- and post-drug administration values (Kruskal-Wallis ANOVA). Significant differences between contralateral and ipsilateral paw withdrawal thresholds are indicated (* $p \leq 0.05$, Mann-Whitney U test). Parthelonide was administered at a dose of [1.5nmol in 50μl], (n=3).


A
Mean Paw Withdrawal Latency (s)
20
15
10
5
0
-5 0
Baseline
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
B
Mean Paw Withdrawal Threshold Force/Unit Area mN/mm2
5000
4000
3000
2000
1000
0
-5 0
Baseline
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Time Post-Injection (min)
Contralateral
Ipsilateral

5.5.3 Regulation of Constitutive Spinal Cord PKA Activity by the Ubiquitin-Proteasome System

In order to obtain a read-out of the function of the ubiquitin-proteasome system in spinal cord, PKA enzymatic activity was monitored, the activity of which is known to be regulated through degradation of the regulatory subunits by the proteasome (Hegde et al., 1997; Chain et al., 1999). Degradation of regulatory subunits, leaving a relative excess of unrestrained catalytic subunits is thought to lead to an elevated level of constitutive enzyme activity, which can be monitored by ex vivo enzyme assays (Roskoski, 1983). Table 3 shows that the fraction of PKA activity that was constitutive was elevated in spinal cord ipsilateral to CCI, and that this elevation was prevented by local administration of the selective proteasome inhibitors epoxomicin and MG-132 to the spinal cord. This matches other molecular and physiological data in suggesting that the activity of the ubiquitin-proteasome system is elevated in response to nerve injury.

Table 5.2. Effects of Topical Administration of the Proteasome Inhibitors Epoxomicin and MG-132 on Constitutive Activation of Protein Kinase A (PKA) Induced by CCI

Following topical administration under anaesthesia of 500 μl epoxomicin (15 μM), MG-132 (100 μM) or vehicle (0.5% dimethylformamide in saline), relevant spinal segments were removed and hemisected, before homogenisation in cold buffer. Samples were assayed for authentic PKA [^{33}P] phosphotransferase activity under constitutive and maximal cAMP-evoked conditions. Total authentic PKA activity was in the order of 100-200 pmol/min/mg tissue under these conditions and similar between ipsilateral and contralateral CCI tissue. Values are expressed as means ± SEM (n=6-8).

* indicates significantly greater than corresponding contralateral CCI values and normal control values ($p \leq 0.05$), Wilcoxon test and Mann-Whitney U test respectively. † indicates significantly less than corresponding vehicle-treated tissue ipsilateral to CCI ($p \leq 0.05$ Mann-Whitney U-test).

	PKA Activity of Spinal Cord Homogenates (Constitutive Activity as % of Total)		
Topical Administration	**Normal control**	**CCI**	
		Ipsilateral	**Contralateral**
Nil	9.5 ± 1.0	15.7 ± 1.2 *	8.5 ± 0.5
Vehicle	7.9 ± 0.8	14.4 ± 1.5 *†	9.1 ± 0.5
Epoxomicin	8.4 ± 1.0	9.9 ± 0.8 †	8.1 ± 1.1
MG-132	-	7.8 ± 0.2 †	8.6 ± 0.5

5.5.4 Distribution of mRNA for UCH-L1 Within the Spinal Dorsal Horn of Normal and CCI Rats

In accordance with the wide expression of UCH protein throughout eukaryotic cell types and in line with its role in the activity of the multifunctional proteasome pathway, the mRNA for the rat homologue of mammalian UCH neuronal isozyme UCH-L1 was distributed widely in spinal cord of both normal and neuropathic animals. Following CCI of the rat sciatic nerve, changes in the expression of mRNA for UCH-L1 were apparent in the dorsal horn ipsilateral to nerve injury. When compared to contralateral and control values, the total number of neurones positively expressing mRNA for UCH-L1 was significantly increased in the ipsilateral dorsal horn, both mediolaterally in laminae I, II and III and laterally in laminae I and II (Table 5.3, Figure 5.11). Silver grain density was significantly increased mediolaterally in laminae I, II, III and V, and laterally in laminae I, II and III (Figure 5.12). There were no significant differences in either cell counts or silver grain densities between contralateral and normal values. Within the ventral horn of normal animals, labelling of motoneurones was especially intense, but showed no significant change following CCI treatment.

5.5.4.1 Controls

To verify the sensitivity of both the oligonucleotide for UCH-L1 and the assay conditions control experiments were carried out using sections pre-treated with Rnase A (section 2.3.7.8). No positively labelled cells were detected in these spinal cord sections. This treatment produced a low and even background distribution of silver grains equivalent to the non-specific background observed in the positive controls (data not shown).

Figure 5.11 UCH-L1 Subunit mRNA Expression in Lamina I, III and Motoneurones of the Rat Lumbar Spinal Dorsal Horn in CCI (Ipsilateral and Contralateral), Normal and Sham-Operated Rats

Highpower lightfield, black and white photomicrographs showing typical levels of UCH-L1 mRNA expression in the mediolateral area of lamina I and III and motoneurones of rat lumbar spinal dorsal horn (scale bars 10μm). Photomicrographs show typical examples of the expression ipsilateral and contralateral to nerve injury and in normal control rats respectively. Positively labelled neurones were identified by a dense accumulation of silver grains (approximately >5 times background expression) over and around haematoxylin stained nuclei.

Analysis of quantitative densitometry data demonstrated a significant increase in the expression of UCH-L1 subunit mRNA ipsilateral to nerve injury when compared to contralateral and control tissue in the superficial laminae of the dorsal horn (A). However, in motoneurones (B) there was no significant alteration in the expression of UCH-L1 mRNA ipsilateral to nerve injury when compared to contralateral as revealed by cell counts and silver grain density. There were no significant differences between contralateral and control levels of mRNA expression as revealed by cell counts and silver grain density (Table 5.3, Figure 5.12).

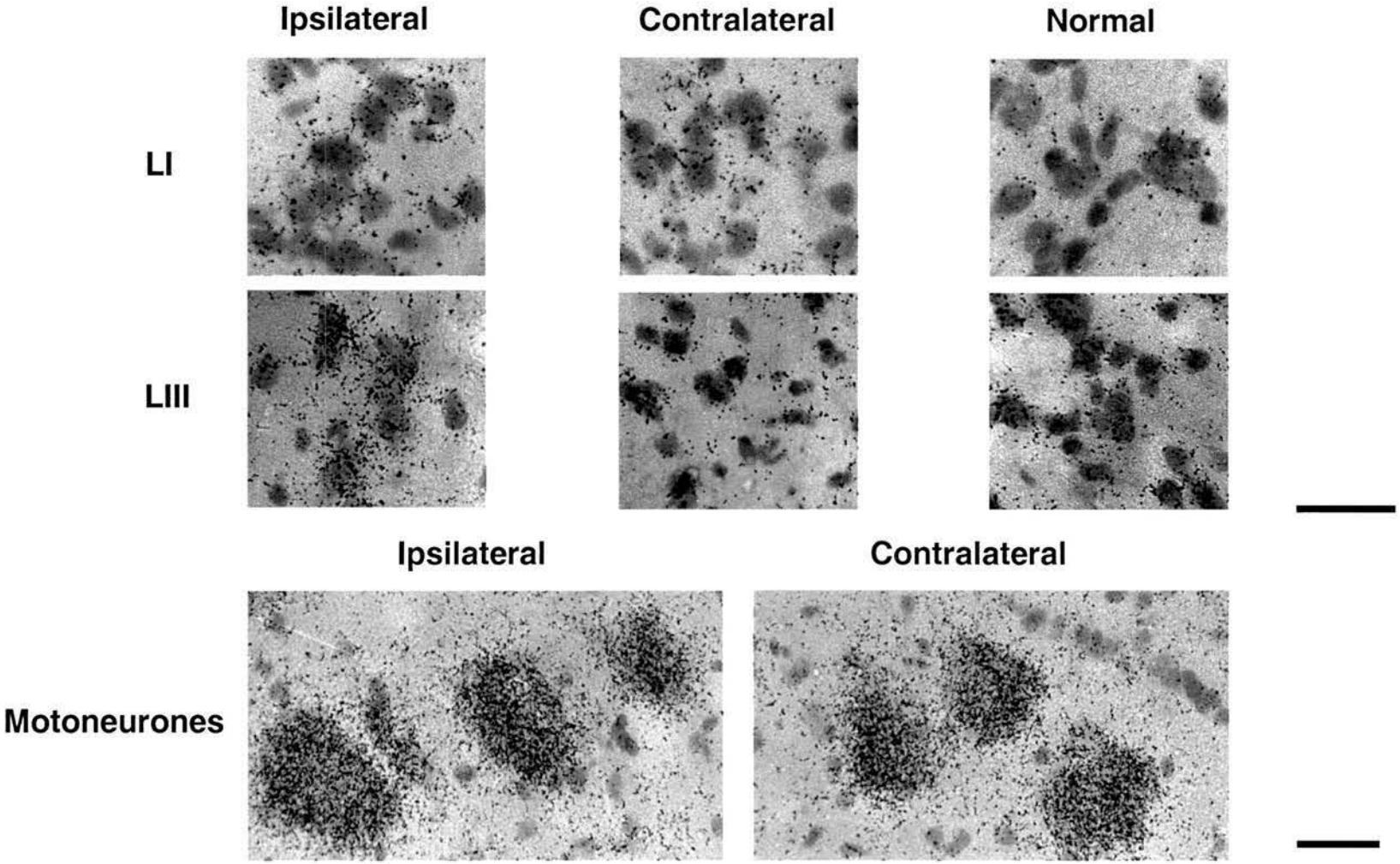

Ipsilateral
Contralateral
Normal
LI
LIII
Ipsilateral
Contralateral
Motoneurones

Table 5.3 Mean Number of Dorsal Horn Neurones Positively Expressing UCH-L1 mRNA

Summary table showing the average number of dorsal horn neurones within a graticule area of 175-175μm^2 positively expressing UCH-L1 mRNA in lamina I, II, III, IV, and V in mediolateral and lateral locations of the dorsal horn.

Values ipsilateral to nerve injury are shown compared to contralateral sham-operated and normal control values.

The mean number of cells expressing mRNA for UCH-L1 was significantly increased ipsilateral to nerve injury in laminae I, II and III (* $p \leq 0.05$; Kruskal-Wallis ANOVA followed by Dunn's post-hoc test) with no significant change in laminae IV and V when compared to contralateral values and to normal, unoperated values. For all laminae analysed there was no significant alteration in the relative expression of UCH-L1 mRNA when comparing contralateral values to normal values.

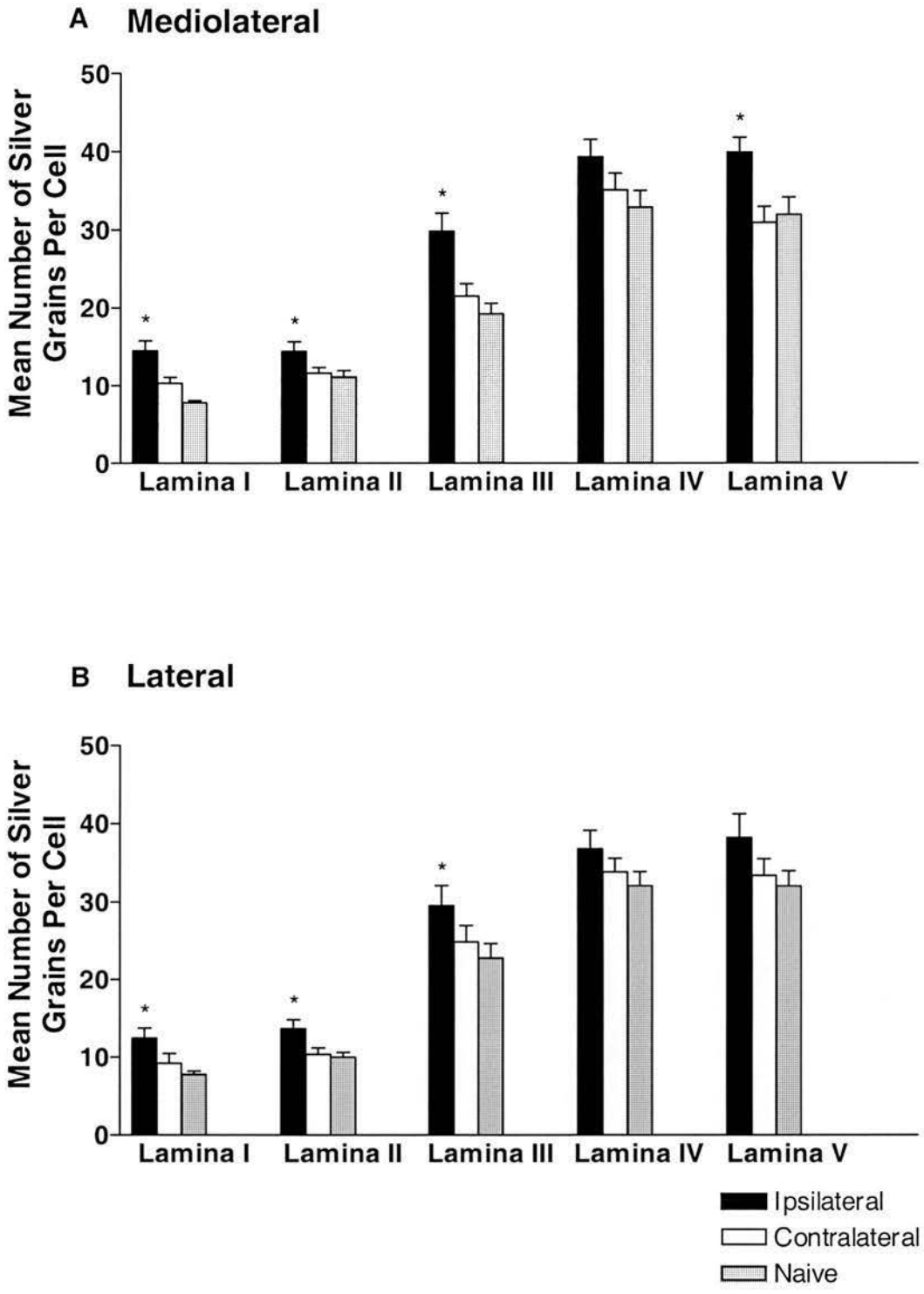
	Cell Counts per 175 x 175 μm^2					
	Mediolateral			Lateral		
	Ipsilateral	Contralateral	Normal	Ipsilateral	Contralateral	Normal
Lamina I	35.5 ± 1.2 *	26.6 ± 0.5	25.9 ± 1.1	32.2 ± 1.4 *	24.4 ± 1.1	22.5 ± 2.5
Lamina II	35.2 ± 1.8 *	25.5 ± 1.2	25.5 ± 1.4	34.2 ± 1.8 *	28.2 ± 2.1	30.1 ± 1.3
Lamina III	37.1 ± 1.9 *	30.5 ± 1.2	30.5 ± 1.5	36.5 ± 3.1	32.5 ± 2.5	35.6 ± 2.2
Lamina IV	42.2 ± 2.2	38.2 ± 3.2	38.8 ± 3.4	41.1 ± 2.1	40.5 ± 1.4	40.1 ± 1.3
Lamina V	38.9 ± 1.4	36.5 ± 1.8	37.5 ± 2.2	32.5 ± 1.2	32.1 ± 0.6	32.4 ± 1.0

Figure 5.12 Mean Silver Grain Density of Dorsal Horn Neurones Positively Expressing UCH-L1 mRNA

Summary histogram showing the mean silver grain density for dorsal horn neurones positively expressing UCH-L1 mRNA in lamina I, II, III, IV and V in mediolateral and lateral locations of the spinal cord.

Values ipsilateral to nerve injury are shown compared to contralateral and normal control values.

The relative silver grain density per positively expressing cell, indicative of the expression of UCH-L1 mRNA, was significantly increased ipsilateral to nerve injury in lamina I, II, III (and mediolateral V) ($*p \leq 0.05$, Kruskal-Wallis ANOVA followed by Dunn's post-hoc test) but was unaltered in lamina IV and (and lateral V) when compared to contralateral values and to normal, unoperated values. For all laminae analysed, there was no significant alteration in the relative expression of UCH-L1 mRNA when comparing contralateral CCI values to normal values.


A Mediolateral
Mean Number of Silver Grains Per Cell
50
40
30
20
10
0
*
Lamina I
Lamina II
Lamina III
Lamina IV
Lamina V
B Lateral
Mean Number of Silver Grains Per Cell
Ipsilateral
Contralateral
Naive

5.5.5 Determination of Protein Levels of UCH-L1 Following CCI

In order to determine whether the increase in UCH-L1 mRNA following CCI leads to an overall increase in the translation of its protein product, Western blot analysis was carried out.

Results were obtained from a total of (12) rats (n=4 CCI, n=4 sham-operated and n=4 normal). Using materials and methods described in detail elsewhere (chapter 2, section 2.3.8.5). Spinal cord samples were homogenised and SDS-PAGE carried out to resolve the protein bands. Following transfer to PVDF, blots were probed using a HRP-conjugated primary antibody specific to UCH-L1 (Table 2.1) and a HRP-conjugated secondary antibody with visualisation by ECL. Blots were also probed with an antibody specific to the ubiquitous cellular housekeeper enzyme GAPDH (Table 2.1). Samples were normalised in relation to GAPDH and values expressed as the percentage GAPDH expression (Figure 5.13).

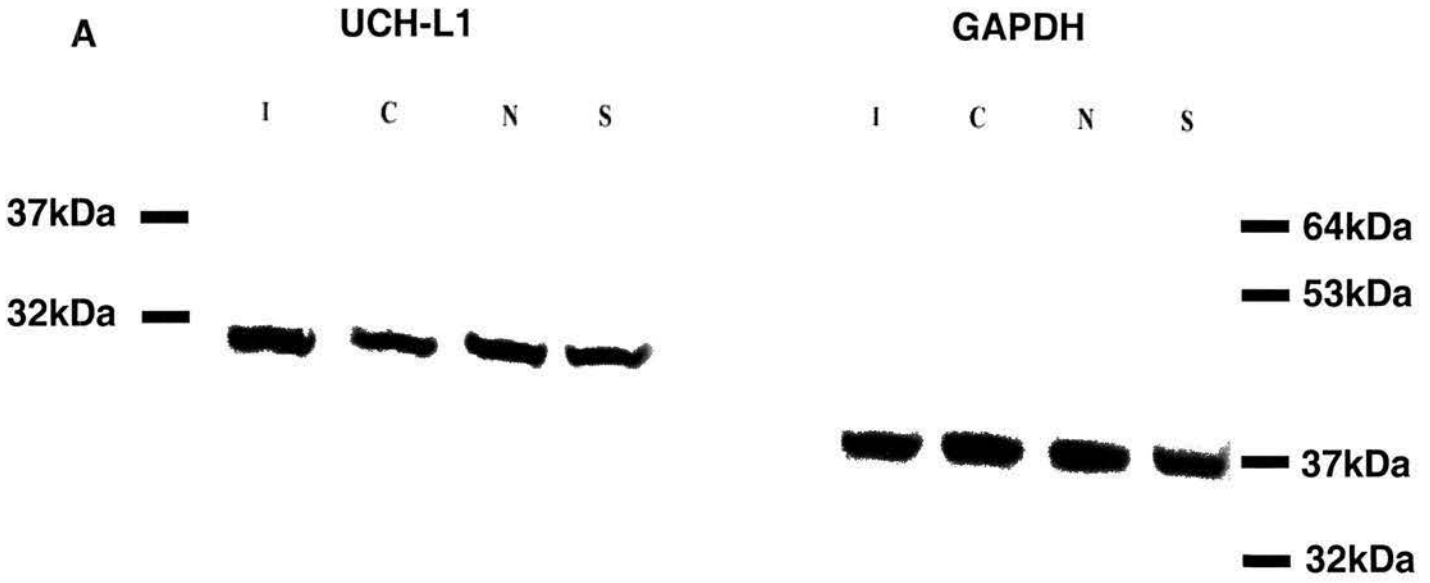
Densitometry of immunoreactive bands revealed an increase in the levels of UCH-L1 in the spinal cord ipsilateral nerve injury when compared to contralateral, sham-operated and normal sample values (Figure 5.13).

Figure 5.13 Western Blot Analysis of UCH-L1 and GAPDH Protein Expression in the Rat Lumbar Spinal Cord in CCI (Ipsilateral and Contralateral), Normal and Sham-Operated Rats

(A) Western blots of hemisected lumbar spinal cord tissue showing UCH-L1 (29kDa) protein expression and the housekeeping protein GAPDH (36kDa). Scanner print-outs from the ECL films show typical examples of UCH-L1 and GAPDH expression within the same lanes. The positions of the molecular weight markers are shown. The expression of UCH-L1 protein ipsilateral to nerve injury (I) appeared consistently greater than that in contralateral (C), normal (N) and sham-operated (S) tissue.

(B) Table represents UCH-L1 expression as a percentage of GAPDH expression in terms of relative grey scale values following quantitative densitometry of ECL films. Data are presented as mean ± SEM (n=4). * $p \leq 0.05$ Student's unpaired t-test compared to contralateral, sham-operated and normal rats.

A



B

	UCH-L1 Immunoreactivity (% of GAPDH)			
	Ipsilateral	Contralateral	Normal	Sham
Mean ± SEM	112 ± 10.6 *	85.4 ± 11.1	88.2 ± 3.0	85.7 ± 7.3

5.6 Discussion

Injury in afferent nerves can elicit their sustained firing and result in phenotypic and functional changes both within dorsal root ganglia and in the dorsal horn of the spinal cord. The resulting state of central sensitisation plays a key role in bringing about the hyperalgesia and allodynia, as well as sensitivity to cold, that characterise neuropathic pain states. PKA is implicated in spinal sensitisation and pain (Cerne et al., 1993; Sluka, 1997) and evidence presented in chapter 4 suggests that PKA may play a role in the central sensitisation that follows peripheral nerve injury. The increase in the activity of PKA demonstrated in the previous chapter may be in part due to an increase in the levels of the catalytic subunits and the parallel decrease in the levels of the regulatory subunit(s) of PKA. A stable change in the R:C ratio may lead to a long lasting and persistent activation of the kinase as seen in long-term facilitation in *Aplysia* sensory neurones (Hegde et al., 1997; Chain et al., 1999), and implies the existence of an important mechanism for regulating the ratio of PKA subunits. Results show that targeted proteolysis by the ubiquitin-proteasome system (involving in part processes that bring about constitutive activation of PKA) is an essential component of the cellular mechanisms underlying neuropathic sensitisation following CCI.

The proteasome appears to contribute to the normal spinal processing of noxious but not innocuous sensory stimuli as dorsal horn neurone firing induced by the application of mustard oil was inhibited by both MG-132 and lactacystin, and responses to innocuous brush evoked activity were unaffected in normal rats. After CCI, previously innocuous stimuli such as brush and cold elicited greatly accentuated responses in dorsal horn neurones. These responses displayed sensitivity to blockade by the proteasome inhibitors MG-132 and lactacystin, which specifically inhibited dorsal horn neurone firing in response to cold, brush and mustard oil-induced activity. Intrathecal administration of the proteasome inhibitors MG-132 and epoxomicin in rats exhibiting peak behavioural changes following CCI, dramatically attenuated reflex withdrawal responses to noxious heat, mechanical stimulation and cold with no effect in normal unoperated rats. The contribution of the ubiquitin-proteasome system to sensitisation was paralleled by increased

expression (ipsilateral to nerve injury) of mRNA for UCH-L1, as analysed using ISHH, and increased levels of immunoreactive UCH-L1 protein as measured by Western blot.

Aspects of the change into a potentiated sensory system that occur following nerve injury appear to parallel the activity-dependent changes that contribute to the persistence of both LTF in *Aplysia* sensory neurones and LTP in central mammalian neurones. This implies that spinal neuropathic sensitisation may constitute a form of pain "memory". Although the precise nature and origin of the alterations in LTF and LTP differ from those here, they may share a number of common features with the central sensitisation that occurs within the spinal cord in response to chronic activation of nociceptive inputs. Activity-dependent synaptic changes associated with altered states of responsiveness and "synaptic memory" have been investigated in LTF (Bailey and Kandel, 1993) and LTP (Bliss and Collingridge, 1993).

The contribution of the ubiquitin-proteasome system here, particularly the ubiquitin recycling enzyme UCH-L1 is consistent with experimental evidence investigating the contribution of the ubiquitin-proteasome system in LTF in *Aplysia* (Hegde et al., 1997; Chain et al., 1999). The steady state elevation of UCH-L1 expression observed here is less than the acute rise seen within 4 hours following LTF in *Aplysia* (Hegde et al., 1997). This may relate in part to the higher basal levels of UCH-L1 in mammalian neurones (Wilkinson et al., 1989). Furthermore, the CCI sensitisation model differs from LTF in that changes in spinal neuronal function develop slowly and are maintained over many days. If optimal UCH-L1 activity is required for operation of the ubiquitin-proteasome system, and this pathway plays a greater functional role following CCI (as demonstrated) then even small changes in UCH-L1 may be functionally significant.

UCH-L1 plays an essential role in the turnover of ubiquitin tags, which is necessary to maintain adequate rates of proteasome-mediated degradation of proteins. The regulatory subunits of PKA are an example of such a known proteasome target. Elevated levels of cAMP lead to the dissociation of the regulatory and catalytic

subunits of PKA, which in turn leads to the activation of PKA. PKA remains active until cAMP returns to basal levels, largely due to the action of phosphodiesterases, and the regulatory and catalytic subunits of PKA reassociate. Degradation of the regulatory subunits of PKA by the ubiquitin-proteasome system is thought to lead to the formation of persistently active PKA that is no longer dependent on elevated levels of cAMP (Bergold et al., 1990; Hegde et al., 1993). These two forms of regulation of PKA activity are likely to operate in concert.

In the LTF model in *Aplysia*, PKA appears to play a key role in increased responsiveness and substantial evidence indicates that an imbalance of PKA regulatory and catalytic subunits, mediated by proteasome degradation of the former, is a crucial factor (Hegde et al., 1993; Chain et al., 1999). The authors speculate that *Aplysia* UCH (*Ap*-UCH) is required for degradation of the inhibitory regulatory subunit of PKA, and that the resultant activation of PKA leads to transcriptional activation of numerous CREB-responsive genes necessary for the persistence of the "learned" behaviour. Thus, the increase in UCH activity upon 5-HT treatment may relieve the inhibition of the regulatory subunit and allow more efficient degradation of the regulatory subunit of PKA, leading to the persistent activation of PKA (Hegde et al., 1993; Chain et al., 1999). The dissociation of the PKA holoenzyme is necessary but not sufficient for degradation by the ubiquitin-proteasome system (Chain et al., 1999). A high concentration of cAMP is initially required to convert the normally stable regulatory subunit into a substrate for ubiquitin ligation, and because in *Aplysia* sensory neurones the levels of cAMP are elevated for relatively short periods, the degradation of the regulatory subunits is an essential stage in the generation of a persistently active kinase (Chain et al., 1999). This matches closely the proteasome-dependent generation of constitutively active PKA observed in the present study ipsilateral to CCI that was prevented by proteasome inhibitors. In the *Aplysia* model, expression of the homologue of UCH-L1 is rapidly increased in the facilitation paradigm (Hegde et al., 1997), again closely paralleling the observations here with neuropathic sensitisation, except that the full development of the sensitised state here occurs over a more prolonged time period.

The rapid and marked inhibition by ubiquitin-proteasome inhibitors of the abnormal behavioural responses both electrophysiologically and by intrathecal administration suggests two things. Firstly that the ubiquitin-proteasome system is involved in the mechanisms underpinning neuropathic sensitisation, and secondly that ubiquitin proteasomal degradation is important at the peak of neuropathy. This data taken with the observation that mRNA and protein levels for UCH-L1 are raised during peak behavioural changes following CCI suggests that protein degradation via the ubiquitin-proteasome system may contribute to the cellular mechanisms that maintain neuropathic sensitisation at the peak of neuropathy.

An obvious target for this degradation may be the regulatory subunit of PKA as discussed. However, there are also several other serine/threonine kinases that are thought to become activated in LTF sensitisation: eg. Ca^{2+}, calmodulin-dependent protein kinase (CaM kinase) (Yamamoto et al., 1999), protein kinase C (PKC) (Sacktor and Schwartz, 1990; Sossin et al., 1994) and mitogen-activated protein kinase (MAP kinase) (Michael et al., 1998). The catalytic subunits of PKA and activated MAP kinase (Martin et al., 1997) may be imported into the nucleus and phosphorylate transcription factors, which then initiate gene expression (Dash et al., 1990; Bartsch et al., 1998), with the resultant upregulation of genes such as the gene encoding UCH-L1. It is an open question as to whether the function of any of these kinases may be directly or indirectly influenced by the proteasome, although it is established for PKC for example, that the proteasome can mediate a pathway for its breakdown (Lee et al., 1996).

An alternative pathway known to be subject to proteasome regulation is that of the transcription factor, nuclear factor kappa B (NF-κB). NF-κB exists in a latent form in unstimulated cells, complexed to the inhibitory protein, I-κB. Inflammation-associated molecules such as cytokines can induce I-κB phosphorylation leading to its ubiquitination and degradation by the proteasome (Karin and Delhase, 2000). Dissociated NF-κB can then regulate the expression of a variety of target genes including the inducible enzymes cyclo-oxygenase-2 and nitric oxide synthase, adhesion molecules, cytokines and neuropeptides (O'Neill and Kaltschmidt, 1997).

Although NF-κB expression decreases acutely following nerve injury (Doyle and Hunt, 1997) NF-κB immunoreactivity has been reported to increase within ipsilateral DRG neurones two weeks after nerve injury (Ma and Bisby, 1998). In pilot experiments with intrathecal administration of the selective NF-κB inhibitor, parthenolide in CCI rats no detectable change was observed in behavioural responses to heat, or mechanical stimuli. Such doses would be predicted to cause clear inhibition of NF-kB-mediated responses suggesting that while certain proteasome targets, like PKA may play an important role in neuropathic sensitisation, others like the NF-κB / I-κB complex may not be important. However, because authentic NF-κB-mediated responses were not evaluated in parallel in these experiments, a role for NF-κB in CCI sensitisation cannot definitively be excluded. Nevertheless, more extensive studies on these and other pathways will be necessary before it is clear how the proteasome plays out its key role in enabling neuropathic sensitisation.

Although direct evidence is lacking, ubiquitin-dependent processes appear to be important in regulating many aspects of signal transduction (Isaksson et al., 1996), including the actions of many receptors such as the receptor tyrosine kinases (Mori et al., 1995), heterotrimeric G protein systems (Madura and Varshavsky, 1994); protein kinases such as PKA (Hegde et al., 1997), and PKC (Lee et al., 1996); transcription factors (Pahl and Baeuerle, 1996) including p53 (Maki et al., 1996), fos/jun (Jariel-Encontre et al., 1997) and as described NF-κB (Rofl et al., 1996; Palombella et al., 1994). Thus, many further targets beyond PKA and NF-κB could potentially contribute to the actions of the ubiquitin-proteasome system here. In summary, results novelly demonstrate that proteasome inhibitors can selectively inhibit neuropathic sensitisation, which is likely to underlie the development of chronic intractable pain following nerve injury. Importantly, proteasome inhibitors attenuate neuropathic allodynia without impairing normal sensory responses to low intensity peripheral stimuli; a particularly advantageous therapeutic profile. Expression of a key enzyme in ubiquitin-proteasome function is increased in spinal dorsal horn ipsilateral to injury, as is the activity of a known target of the complex (PKA), which is expected to display de-regulated constitutive activity following proteasome action (collaborative studies in the laboratory).

These findings suggest that proteasome inhibitors may have therapeutic potential in neuropathic pain. As a result of the diverse cellular roles of the proteasome including the regulation of cell survival and proliferation, proteasome inhibitors are under phase I trials as anti-cancer drugs (Lee and Goldberg, 1998). In many cases, advanced development of tumours leads to local inflammation and pressure trauma to afferent nerves, resulting in a neuropathic component to cancer pain. The present study predicts that in addition to any direct effect on cancer cells, proteasome inhibitors would exert a useful additional role in attenuating the central sensitisation that leads to chronic hyperalgesia and allodynia.

CHAPTER 6: SUMMARY AND CONCLUSIONS

Neuropathic pain due to nerve injury may occur as a result of peripheral tissue damage due to disease or trauma, or may be a direct result of nerve transection, crushing or constriction. The chronic pain states which develop include spontaneous pain, hyperalgesia and allodynia, and they may persist long after the initial injury has healed (Scadding, 1984). These abnormal pain states show a reduced sensitivity to classical opioid analgesics (Arner and Meyerson, 1988; Mao et al., 1995). The mechanisms underlying the development of neuropathic pain are poorly understood, but inflammatory mediators released at the site of nerve injury (Tracey and Walker, 1995) and central changes within the spinal cord (Woolf and Costigan, 1999), are all thought to contribute. Several studies have implicated the $VPAC_2$ receptor in the modulation of nociceptive processing in neuropathic pain (eg. Dickinson et al., 1999). The signal transduction mechanisms (via G protein-coupled receptors) involving the activation of adenylate cyclase and the subsequent activation of PKA, downstream of VPAC / PAC receptors have also been implicated in the underlying mechanisms of nociceptive sensitisation (Coderre et al., 1993; Malmberg, 2000). The present study simultaneously addressed the role of the $VPAC_2$ receptor, the cAMP-dependent signal transduction cascade and the persistent activation of PKA by the ubiquitin-proteasome system in neuropathic sensitisation following CCI.

6.1 The Role of the $VPAC_2$ Receptor in Neuropathic Pain

The current investigation utilised behavioural reflex techniques to further elucidate the specific role of the $VPAC_2$ receptor in neuropathic sensitisation following peripheral nerve injury. There is a growing body of evidence implicating the $VPAC_2$ receptor in nociceptive processing (Dickinson and Fleetwood-Walker, 1999). However, the precise involvement of the $VPAC_2$ receptor in the injury-induced sensitisation following peripheral nerve injury has not yet been adequately addressed.

This was initially assessed using wild-type mice and mutant mice lacking the $VPAC_2$ receptor ($VPAC_2R^{(-/-)}$). CCI was carried out in wild-type and $VPAC_2R^{(-/-)}$ mice and the development and maintenance of the abnormal behavioural responses that occur

following CCI were compared. To further assess the contribution of the $VPAC_2$ receptor to the maintenance of the abnormal behavioural responses within the spinal dorsal horn at the peak of neuropathy, a selective antagonist of the$VPAC_2$ receptor was administered intrathecally at the level of the lumbar spinal cord in wild-type and $VPAC_2R^{(-/-)}$ mice exhibiting peak behavioural changes following CCI. In wild-type mice, intrathecal injection of the $VPAC_2$ receptor antagonist significantly reversed the accentuated reflex withdrawal responses that develop following CCI that are believed to be indicative of neuropathic pain. This inhibition was specific to the ipsilateral limb and showed no effect on the contralateral limb or in the $VPAC_2R^{(-/-)}$ mice. Subsequently, the effects of intrathecal administration of a selective agonist of the $VPAC_2$ receptor were examined in both wild-type and $VPAC_2R^{(-/-)}$ mice. Following injection of the $VPAC_2$ receptor agonist, the latency/threshold for reflex withdrawal responses to noxious heat and innocuous mechanical stimuli decreased progressively and reached peak behavioural change (ipsilateral vs. contralateral) at ~ day 12 post-operatively. In $VPAC_2R^{(-/-)}$ mice however, the decreases in reflex withdrawal latency/threshold following CCI were dramatically attenuated when compared to wild-type mice. These results taken together with previous findings (Dickinson and Fleetwood-Walker, 1999) suggest that there may be an increased involvement of the $VPAC_2$ receptor in spinal sensory processing following nerve injury that may participate in the development and maintenance of neuropathic pain. In the present behavioural study, the sensitisation attenuating effects of the $VPAC_2$ receptor antagonist and of the $VPAC_2$ receptor knockout were more marked on responses to heat than to mechanical stimuli. This suggests that $VPAC_2$ receptor antagonists might be useful to target selectively the C fibre-mediated hyperalgesia seen in neuropathic pain.

This project provides new insights into the role of $VPAC_2$ receptor within the spinal dorsal horn, and underlines the potential importance of the $VPAC_2$ receptor antagonists as new analgesic agents for use in currently intractable neuropathic pain states. The $VPAC_2$ receptor is clearly very different from that of other modulators of sensory processing, such as the opioid and tachykinin receptors (Fleetwood-Walker et al., 1988; 1993) as neuropathic pain appear to be less responsive to classical opioid

analgesics than are acute or inflammatory pain states (Arner and Meyerson, 1988; Mao et al., 1995).

6.2 The Role of Cyclic-AMP Dependent Protein Kinase in Neuropathic Pain

The cAMP / PKA signal transduction pathway was investigated using behavioural and molecular techniques to reveal its contribution to the central sensitisation that manifests following nerve injury. Activation of PKA by increased levels of cAMP downstream of G protein-coupled receptor activation has been implicated in the mechanisms underpinning persistent nociception in alternative centrally and peripherally-mediated models (Coderre et al., 1993; Sluka 1997; Taiwo et al., 1999), as well as in parallel mechanisms of synaptic plasticity (Cerne et al., 1992; 1993; Hegde et al 1993). However, the importance of PKA in central neuropathic sensitisation following peripheral nerve injury has not been investigated.

This issue was initially assessed by observing the effect of intrathecal administration of selective PKA inhibitors on the abnormal behavioural responses in rats at the peak of neuropathy. The effects of the selective PKA antagonists on reflex withdrawal responses to noxious heat, innocuous mechanical stimulation and innocuous cold were assessed. A Western blot study was used to investigate the levels of expression of the regulatory and catalytic subunits of PKA within the spinal dorsal horn, and to ascertain as to whether the expression of any of these subunits were altered following CCI. Finally, a complementary in situ hybridisation histochemistry study was carried out to investigate the distribution of mRNA for the regulatory and catalytic subunits of PKA within the spinal dorsal horn, and to ascertain as to whether their expression was altered following CCI. Results from chapter 4 demonstrate that PKA inhibitors act to selectively reverse neuropathically sensitised responses, markedly reversing the accentuated responses to heat and mechanical stimuli following nerve injury. Taken together with the increased expression of mRNA and protein levels for the catalytic subunits (Cα and Cβ) the observations suggest that persistent activation of PKA following nerve injury may play a key role in the sensitised responses to heat and mechanical stimuli. This suggests that there may be an increase in the activity of

PKA following nerve injury that may participate in the development and maintenance of neuropathic pain. The continued sensitivity to an inhibitor of cAMP-dependent PKA activation suggests that ongoing activity of relevant receptors such as the VPAC / PAC receptors may play a key role in the ongoing maintenance of the neuropathic pain state.

The potential of the mechanisms of action of PKA as a target from novel neuropathic analgesics may be promising in the longer term. However, the idea of targeting PKA isoforms themselves for therapeutic value would not be a sensible approach as the ubiquitous nature and the great variety of important physiological roles that PKA is implicated in would speak against PKA being a suitable target in itself. However, mechanisms uncovered in this project may facilitate further research on PKA related targets which may underpin neuropathic sensitisation.

6.3 The Role of the Ubiquitin–Proteasome System in Neuropathic Pain

Persistent activation of PKA is widely believed to underpin the mechanism of long term memory formation in *Aplysia* (Greenberg et al., 1997) and in the mammalian hippocampus (Frey et al., 1993). Increasing evidence has suggested that targeted protein degradation of the regulatory subunits of PKA by the ubiquitin-proteasome system, thus leading to a persistently active kinase, may be responsible (Hegde et al., 1993; 1997; Chain et al., 1999).

In light of the interesting findings in chapter 4 implicating persistently active PKA in the underlying mechanisms of central neuropathic sensitisation, electrophysiological, behavioural and molecular biological techniques were adopted to address the role of the ubiquitin-proteasome system in central neuropathic sensitisation following peripheral nerve injury. Firstly, an electrophysiological study was carried out using selective antagonists of the ubiquitin-proteasome system. The effects of these antagonists were investigated on the sustained neuronal activity of individual dorsal horn neurones induced by sensory stimuli in normal rats and in neuropathic rats at the peak of neuropathy. Complementary experiments investigating the effects of intrathecal administration of selective ubiquitin-proteasome inhibitors on the

abnormal reflex responses that develop in rats at the peak of neuropathy following CCI were carried out confirming selective attenuation of the neuropathically sensitised responses. A Western blot study was used to investigate the levels of expression of a key enzyme of the ubiquitin-proteasome system, UCH-L1 within the spinal dorsal horn, and to ascertain as to whether the expression of this enzyme was altered following CCI. Finally, a complementary ISHH study was carried out to investigate the distribution of mRNA for UCH-L1 within the spinal dorsal horn, and to ascertain whether the expression was altered following CCI. Results from chapter 5 suggest that in the normal state the ubiquitin-proteasome system inhibitors were selectively antinociceptive (markedly inhibiting only the sustained C-fibre activity induced by topical application of the chemical irritant mustard oil). In contrast, the proteasome appeared to play an enhanced role following CCI (with inhibitors markedly attenuating not only the C fibre activity-induced by topical application of mustard oil, but now also the innocuous brush evoked activity). The findings that intrathecal administration of proteasome inhibitors markedly reverse the sensitised responses to heat and mechanical stimuli following nerve injury, together with the marked upregulation ipsilateral to nerve injury of mRNA and protein levels for the ubiquitin recycling enzyme UCH-L1 suggest that protein degradation (via the ubiquitin-proteasome pathway) may underpin the persistent activation of PKA and the sensitivity to heat and mechanical stimuli that follow nerve injury.

It has been demonstrated that proteasome inhibitors can selectively inhibit neuropathic sensitisation, which is likely to underlie the development of chronic intractable pain following nerve injury. Importantly, proteasome inhibitors attenuate neuropathic allodynia without impairing normal sensory responses to low intensity peripheral stimuli; a particularly advantageous therapeutic profile. Expression of a key enzyme in ubiquitin-proteasome function (UCH-L1) is increased in spinal dorsal horn ipsilateral to injury, as is the activity of a known target of the complex (PKA), which is expected to display de-regulated constitutive activity following proteasome action. Proteasome dependent activation of PKA in neuropathic sensitisation closely parallels that in LTF in *Aplysia*. Proteasome inhibitors are under phase I trials as anti-cancer drugs (Lee and Goldberg, 1998). In many cases, advanced development

of tumours leads to local inflammation and pressure trauma to afferent nerves, resulting in a neuropathic component to cancer pain. The present study predicts that in addition to any direct effect on cancer cells, proteasome inhibitors would exert a useful additional role in attenuating the central sensitisation that leads to chronic hyperalgesia and allodynia.

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